From the Department of Neuroscience
Karolinska Institutet, Stockholm, Sweden

ON NOGO RECEPTORS, PLASTICITY AND LASTING MEMORIES

Tobias Karlsson

Karolinska Institutet

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ABSTRACT

The central nervous system has very limited ability to repair itself after injury but has been shown to exhibit remarkable experience-driven structural plasticity at the synaptic level. These changes correlate strongly with behavioral performance and are thought to underlie learning and memory. MAG, OMgp and Nogo are a group of neurite growth inhibitors present in CNS myelin and exerting effects by binding to a common receptor, NgR1. Because Nogo is also expressed by neurons and NgR1 is exclusively expressed in neurons and downregulated by activity, NgR1 may regulate plasticity. For local synaptic plasticity to occur one requirement could be that NgR1 signaling decreases. In fact, NgR1 is rapidly downregulated in key areas associated with plasticity such as hippocampus in a variety of different models of neuronal activity. When NgR1 is knocked out, mice show increased plasticity and continue to demonstrate ocular dominance shift plasticity in the visual cortex into adulthood.

The aim of this thesis was to further investigate how the Nogo-system is regulated and to evaluate how it may influence memory formation. It is confirmed that increased neuronal activation (using Kainic acid) causes rapid downregulation of NgR1 and a more detailed time course of this effect is presented. The NgR1 downregulation might be further potentiated through an increase of the endogenous NgR1 antagonist LOTUS in the dentate gyrus. In contrast to NgR1, both NgR2 and NgR3 were mostly upregulated following Kainic acid injection. As the spectrum of known ligands for the different NgRs differ, this could result in a change in responsiveness to different inhibitor types and might favor increased local plasticity while keeping plasticity at the level of non-terminal axons and main dendrites in check.

We created a mouse with inducible overexpression of NgR1 in forebrain neurons (MemoFlex) to test the hypothesis that reduced NgR1 levels are important for memory formation. We found that while NgR1 overexpression does not impair 24 h memory and day to day learning, it significantly impairs formation of lasting memories as shown in both the Morris water maze and a passive avoidance test. By turning the NgR1 transgene off (by doxycycline) at different time points, we were able to demonstrate that NgR1 downregulation-dependent memory consolidation occurs during the first 7-9 days after a memory-forming event. MemoFlex mice exhibited increased sensitization to amphetamine (that could be normalized by turning off the transgene), as if they were unable to "learn" and hence cope with amphetamine injections lasting for as long as a week. When retested after 110 days MemoFlex mice showed a significant reduction of "sensitization memory" compared to their performance at the end of the sensitization period. Control mice tended to increase sensitization during the withdrawal period. Mice lacking NgR1 did not differ from control mice with respect to a week-long amphetamine sensitization. Analysis of spine density and subtypes in the cingulate gyrus and the shell of nucleus accumbens indicated that NgR1 overexpression has significant effects on spine dynamics.

NgRs have also been shown to affect the deposition and formation of Aβ-plaques in mouse models of Alzheimer's disease. To test if NgR1 overexpression would affect plaque deposition we crossed MemoFlex mice with plaque-forming APPswe/PSEN1 mice. While we could not see any significant change in plaque formation in APPswe/PSEN1 mice with NgR1 overexpression, they were significantly impaired in the Morris water maze. The fact that performance was significantly decreased while plaque formation was unaffected could result from impairment of compensatory synaptic plasticity that otherwise occur in plaque-forming mice.

Taken together, these studies suggest a role for the Nogo-system, and in particular NgR1, for the formation of lasting memories.

LIST OF PUBLICATIONS

I. Karlén A, Karlsson TE, Mattsson A, Lundströmer K, Codeluppi S, Pham TM, Bäckman CM, Ogren SO, Aberg E, Hoffman AF, Sherling MA, Lupica CR, Hoffer BJ, Spenger C, Josephson A, Brené S, Olson L (2009)
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III. Karlsson TE, Åberg E, Karlén A, Lindskog M, Olson L, Josephson A, Brené S. Nogo-receptor 1 regulates drug-induced functional and structural brain plasticity. Manuscript

IV. Karlsson TE, Koczy J, Brené S, Olson L, Josephson A. Activity induced regulation of Nogo receptors (1-3) and LOTUS mRNA in mouse brain. Manuscript

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CA	Cornu Ammonis
CNS	Central nervous system
CSPG	Chondroitin sulfate proteoglycans
CST	Corticospinal tract
Dox	Doxycycline
EPSP	Excitatory postsynaptic potential
fEPSP	Field EPSP
GPI	Glycosylphosphatidylinositol
H	Hour(s)
i.p	Intra peritoneal
ISH	In situ hybridization
LRR	Leucine rich repeats
LTD	Long term depression
LTP	Long term potentiation
MAG	Myelin associated glycoprotein
MAI	Myelin associated inhibitor
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MTL	Medial temporal lobe
MWM	Morris water maze
NgR	Nogo-receptor
OMgp	Oligodendrocyte myelin glycoprotein
PNS	Peripheral nervous system
RAWM	Radial arm water maze
SEM	Standard error of the mean

INTRODUCTION

A phenomenon that has received a substantial amount of attention within the field of neuroscience is the fact that the central nervous system (CNS) is highly inhibitory to neuronal outgrowth and regeneration. It has been long known from transplantation experiments that CNS neurons can extend neurites into grafts from the peripheral nervous system (PNS) (Benfey and Aguayo, 1982; Richardson et al., 1984; So and Aguayo, 1985). This is interesting as it shows that CNS neurons possess the ability to grow but require a different environment to be able to do so. In contrast, PNS neurons that have been shown to exhibit rather good regeneration lose this function when exposed to material from the CNS (Aguayo et al., 1978; Weinberg and Spencer, 1979). Hence while PNS appears to enhance neuronal outgrowth, the CNS instead actively impairs it.

NERVE GROWTH INHIBITORY MOLECULES OF CNS MYELIN

In 1988 it was shown that two protein fractions of 35 and 250 kDa found in CNS myelin (but not in PNS myelin) were inhibitory for neuronal outgrowth (Caroni and Schwab, 1988b). It was also shown that two antibodies, IN-1 and IN-2, raised against the 250 kD and 35 kD protein respectively, could bind to both the 35 and the 250 kDa fragment (suggesting that they share a functional domain) and counteract the inhibitory effect of CNS myelin (Caroni and Schwab, 1988a). It was for instance shown that neurons in optic nerve explants could grow over 3 mm, something that only occurred occasionally when a control antibody was used. The inhibitory substance isolated from myelin, Nogo, was cloned in 2000 (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000) using a sequence from proteolytically digested Nogo published earlier (Spillmann et al., 1998). Nogo is a member of the Reticulon family and exists in 3 different isoforms, Nogo-A, B and C. Of these isoforms Nogo-A has the most specific expression in CNS, while Nogo-B and C are also strongly expressed in other tissues. For instance, Nogo-B has been shown to have effects in endothelial cells (Acevedo et al., 2004; Miao et al., 2006; Yu et al., 2009) while the functions of Nogo-C are less well documented. Another neuronal outgrowth inhibitor, Myelin-associated glycoprotein (MAG), was identified early and found to be highly expressed in myelin (McKerracher et al., 1994; Mukhopadhyay et al., 1994).

Nogo-A (by far the most studied form) contains an extracellular loop, Nogo-66 (Consisting of 66 amino acids) (GrandPre et al., 2000) that can mediate part of the inhibitory effects of Nogo-A. Apart from the effects of Nogo-66, the N-terminal part of Nogo is also a potent inhibitor of outgrowth. Thus antibodies that bind to the N-terminal part of Nogo-A can decrease the outgrowth inhibitory effects of Nogo-A (Chen et al., 2000; Oertle et al., 2003) and expression of only the N-terminal part was found to be inhibitory for outgrowth (Prinjha et al., 2000). It was recently shown that the inhibitory domain Nogo-A Δ20 (in the central Nogo-A specific region) can be internalized and be retrogradely transported to the cell body and there affect RhoA and CREB activity (Joset et al., 2010). Nogo-A was first identified as an inhibitor preferentially expressed in myelin but later studies showed that it was also expressed in neurons in several regions of both the maturing and adult CNS. These regions include the cerebral cortex and the hippocampus (Huber et al., 2002; Josephson et al., 2001;

Lee et al., 2008; Wang et al., 2002c), supporting a role for Nogo-A not only in myelin-neuron interaction but also in neuron-neuron interaction. A third myelin associated inhibitor (MAI) was later identified and named Oligodendrocyte Myelin glycoprotein (OMgp) (Kottis et al., 2002).

THE NOGO-66 RECEPTOR

The first receptor for Nogo-A was found by screening for proteins that could bind to the Nogo-66 domain (Fournier et al., 2001) and was therefore named the Nogo-66 receptor (NgR), While Nogo-66 is sufficient to induce binding, the affinity can be increased further by including parts of the C-terminal domain of Nogo-A (Hu et al., 2005) and inhibition by NgR1 is more potent when a longer fragment (Nogo-22) is used instead of Nogo-66 (Huebner et al., 2011). It was later shown that there are two homologous Nogo receptors now commonly referred to as NgR2 and NgR3 (Barton et al., 2003; Lauren et al., 2003; Pignot et al., 2003). Hence what used to be called NgR is now most often referred to as NgR1. NgR1 was shown to consist of a signal sequence followed by 8 leucine rich repeats (LRR) domains, a LRR carboxy terminal flanking domain with a final unique region containing a GPI anchor (Fournier et al., 2001). Shortly after the discovery that NgR1 could mediate some of the inhibitory effects of Nogo-A it was also shown that MAG can bind to and induce growth cone collapse through NgR1 (Domeniconi et al., 2002; Liu et al., 2002). MAG had previously been shown to induce RhoA activation through p75 (Yamashita et al., 2002) and it was suggested that this could possibly involve sialic acid dependent binding of MAG to the gangliocide GT1b. However in the studies identifying NgR1 as a receptor for MAG it was shown that MAG binding to NgR1 was sialic acid independent (Domeniconi et al., 2002; Liu et al., 2002); this together with the fact that the inhibitory function of MAG was diminished after treatment with PI-PLC suggested that binding to GT1b was not required for MAG function (however the independence of sialic acid has been questions (Robak et al., 2009)). Of note, the two papers did however come to different conclusions concerning if MAG and Nogo-66 competed in binding to NgR1. One paper showed a dose dependent decrease of binding of MAG (Domeniconi et al., 2002) with increasing concentrations of Nogo-66 and the other paper showed no effect (Liu et al., 2002). In a screen to identify GPI-linked myelin inhibitors, OMgp was shown to be able to bind to NgR1 (Wang et al., 2002b) and similarly to Nogo and MAG, OMgp can signal through NgR1 and induce growth cone collapse (Wang et al., 2002b). While it is clear that MAIs can induce growth cone collapse by binding to NgR1, the roles of MAIs on nerve fiber growth and other aspects of axon functions are not fully understood (Chivatakarn et al., 2007; Huebner et al., 2011).

Following the discovery of the myelin-derived ligands, NgR1 has been shown to bind to several other ligands. For instance, NgR1 can bind to B-lymphocyte stimulator (BLYS), part of the immune system, shown to have inhibitory effects on neurite outgrowth from dorsal root ganglion neurons (Zhang et al., 2009). Furthermore, NgR1 appears to be involved in EGFR signaling and its effects on neuronal outgrowth (Koprivica et al., 2005). However, little is known about the significance of that interaction. The function of NgR1 receptor signaling was further complicated when it was shown that phosphorylation of NgR1 by extracellular casein kinase 2 makes it unable to bind to MAG, OMgp or Nogo-66 (Takei, 2009).

ENDOGENOUS NGR1 INHIBITORS

NgR1 function is not only modulated by the different activating ligands discussed above. It has been shown that the nervous system expresses several endogenous NgR1 antagonists. The first such endogenous inhibitor of Nogo-66 - NgR1 signaling to be found was Leucine-rich glioma inactivated 1 (LGI1) (Thomas et al., 2010). It was also shown that NgR1 could enhance the binding of ADAM22 to LGI1. This suggests a new pathway through which NgR1 could influence synaptic plasticity as LGI1 and ADAM22 interaction have been shown to affect dendritic pruning and synaptic function (Fukata et al., 2006; Zhou et al., 2009).

Searching for molecules that could affect the development of the lateral olfactory tract it was found that Lateral Olfactory Tract Usher Substance (LOTUS) could inhibit the binding of Nogo-A to NgR1. Thereby local Nogo-NgR1 growth inhibition can be blocked (Sato et al., 2011), as mediated through the C-terminal domain of LOTUS (Kurihara et al., 2012).

Another inhibitor of NgR1 is the secreted protein Olfactomedin 1 (Nakaya et al., 2012), that is able to decrease the binding of NgR1 to its co-receptor and thereby inhibit NgR1 function. The expression of Olfactomedin 1 co-localizes with that of NgR1 in the dentate gyrus and CA3 of the hippocampus, both areas shown to be highly plastic. This raises the possibility that secretion of Olfactomedin 1 can be one way to overcome NgR1 induced growth inhibition. Thus far, these three inhibitors appear to be specific for NgR1 and not affect the other NgRs.

NGR1 AND ITS CO-RECEPTORS

As NgR1 is attached to the cell membrane by a GPI link, it lacks a cytoplasmic domain and requires co-receptors to transduce a signal into the cell. The first co-receptor to be identified for NgR1 was the low affinity-neurotrophin receptor p75 (Wang et al., 2002a) and it was shown that together with NgR1, p75 could mediate effects of Nogo-A, MAG and OMgp (without affecting the affinity with which these ligands bind to NgR1). When MAG was added to the binding assay the interaction between NgR1 and p75 increased, suggesting that when a ligand binds to NgR1 it increases its affinity for p75 (Wang et al., 2002a). In the same year it was shown that when MAG binds to p75 the activity of RhoA will increase (Yamashita et al., 2002) and subsequent studies have continued to implicate RhoA as a downstream effector of Nogo-signaling (reviewed in (Nash et al., 2009)). It was later shown that while NgR1 and p75 could induce growth cone collapse in several different cell types, in some it required a second co-receptor to function in the form of Lingo-1 (Mi et al., 2004), possibly assisted by gangliosides (Saha et al., 2011). Lingo-1 is specifically expressed in neurons of the CNS and the expression decreases caudally and overlaps well with the expression of NgR1 (Barrette et al., 2007; Josephson et al., 2002). The expression of Lingo-1 is high around birth and decreases until prenatal day 8 (P8) but the expression remains substantial and stays at a similar level as that seen at day 8 into adulthood. Compared with the expression of Lingo-1 and NgR1, the expression of p75 is rather limited and several neuronal sub-types that are strongly inhibited by myelin do not express p75 (Chao, 2003; Roux and Barker, 2002). In 2005 two groups published studies that identified TROY (Park et al., 2005; Shao et al., 2005) as a new co-receptor for NgR1. TROY (Tumour necrosis factor-α (TNFα) receptor superfamily member 19) was found to be able to form a

complex with NgR1 and Lingo-1 even without p75. NgR1/Lingo-1/TROY expressing Cos7 cells induce RhoA activation in a similar manner as NgR1/Lingo-1/p75 cells, suggesting that TROY can replace the function of p75. Furthermore, overexpression of full length TROY in insensitive p75-/- neurons made them sensitive to growth cone collapse by Nogo-66 (Park et al., 2005). Of note, while p75 expression is high prenatally, the expression decreases sharply around birth. In contrast, TROY is expressed at rather high levels until p8, and drops to somewhat lower levels but retains higher expression than p75 into adulthood. This correlated much better with the expression patterns of Lingo-1 and NgR1 (Shao et al., 2005). In both studies TROY was found in neuronal cells of the CNS (Park et al., 2005; Shao et al., 2005). A later study investigating TROY expression in the developing and postnatal CNS found that TROY was mainly expressed in radial glia like cells during development and in astrocytes but not in post natal neurons (Hisaoka et al., 2006). Barrette and colleagues (Barrette et al., 2007) showed, using in situ hybridization, very low expression of TROY in most regions of CNS except for the hippocampal formation (but not in the dentate gyrus), islands of Calleja, laterodorsal tegmental nucleus and zona incerta. Hence it is still possible that more co-receptors remain to be found for NgR1.

NGR2 AND NGR3

There are two homologous receptors to NgR1, NgR2 and NgR3; that show largely overlapping expression patterns with NgR1 but with some significant differences (Barrette et al., 2007; Lauren et al., 2003; Pignot et al., 2003). For instance, NgR2 shows significant mRNA expression in striatum (caudate nucleus) which is not true for NgR1 or NgR3 (however NgR1 protein (Venkatesh et al., 2005) is expressed in striatum, possibly in axons projecting from other NgR1 expressing areas). NgR3 lacks or has a very low expression in diencephalon while both NgR1 and NgR2 exhibit significant expression. Even though NgR2 and NgR3 show a strong homology to NgR1, their binding affinity to myelin inhibitors is significantly different. NgR2 shows no affinity for Nogo-A and OMgp but can bind to MAG with high affinity (Lauren et al., 2007; Robak et al., 2009; Venkatesh et al., 2005). NgR3 on the other hand, lacks affinity for any of the three myelin inhibitors (Venkatesh et al., 2005). NgR2 has also been shown to bind to Fbs1, an ubiquitin ligase associate protein (Kern et al., 2012). The functional relevance of this interaction is yet to be determined but could suggest a way to regulate NgR2 protein levels. While NgR3 lacks affinity to MAG, OMgp and Nogo, it can bind to chondroitin sulfate proteoglycans (CSPG) (Dickendesher et al., 2012) and this is also true for NgR1. CSPGs are a large family of extra cellular matrix proteins that have been shown to be inhibitory to neuronal outgrowth at least partly through activation of RhoA. The formation and deposition of extracellular CSPGs increases after injury, especially in astrocytes that help to form a glial scar around the injury.

STRUCTURE OF NGR1 AND LIGAND BINDING

An in-depth characterization of ligand binding to NgR1 and NgR2 was carried out by Laurén and colleagues (Lauren et al., 2007) and confirmed the previous finding that NgR1 can bind to OMgp, MAG and Nogo-66 and that MAG could bind to NgR2 with high affinity. On NgR1 the core binding site of all three myelin associated inhibitors was found on the concave side of the LRR domain that had previously been shown to be required for ligand binding. There was some difference in binding sites between the

different ligands so that while the core domain is similar, the exact interaction between the different inhibitors appears to differ.

EXPRESSION OF MYELIN INHIBITORS AND RECEPTORS

Barrette and colleagues (Barrette et al., 2007) analyzed the expression of receptors for myelin inhibitors in CNS of mice and together with previous studies (Hunt et al., 2002; Josephson et al., 2002; Zheng et al., 2005) gives an overall consistent picture of the expression patterns of these molecules. NgR1 is expressed in large parts of the telencephalon with the most notable exceptions being striatum and the septal region. In diecephalon the expression is high in thalamus while expression was not detected in hypothalamus. In the brainstem NgR1 expression is more limited but present in several areas that project to the spinal cord.

NgR2 is expressed in almost all areas where NgR1 is expressed with the biggest exception being the islands of Calleja (no NgR2 expression) and striatum (no NgR1 expression) where expression patterns differ. Lingo-1 has an almost ubiquitous expression in the brain and covers all areas that show expression of NgR1 and NgR2. It is also expressed in areas without Nogo-receptors (Barrette et al., 2007; Okafuji and Tanaka, 2005), suggesting that it might have functions outside of myelin mediated outgrowth inhibition. The expression of both p75 and TROY is rather limited compared to NgR1, NgR2 and Lingo-1 and they are not expressed (or expressed at very low levels) in large areas of the cerebral cortex. Expression is also lacking in most of diencephalon and the brain stem.

Nogo-A is strongly expressed in myelin throughout life. When it comes to neuronal expression of Nogo-A, it is rather strong during development and decreases in adulthood. However, expression remains high in some neuronal populations such as those of the olfactory bulb, pyramidal neurons in cortex and neurons of the hippocampus (Huber et al., 2002; Hunt et al., 2003; Hunt et al., 2002; Josephson et al., 2001; Mingorance et al., 2004). As all these regions are known for being highly plastic, the high expression of Nogo-A in these regions is interesting. The cellular distribution of Nogo-A was analyzed by Wang and colleagues (Wang et al., 2002c) and Nogo-A was found to be expressed both in the outer circumference of myelin sheaths and in the inner adaxonal membrane, while NgR1 protein was not found in myelin but was specific to neurons. It has also been shown that all iso-forms of Nogo (A,B,C) are expressed on the cell surface and that they can interact with each other (Dodd et al., 2005). NgR1 protein has been found both pre- and postsynaptically, there were no examples of a synapse with NgR1 at both the pre- and postsynaptic membrane; suggesting that pre- or postsynaptic distribution of NgR1 is regulated (Wang et al., 2002c). Nogo-A in neurons is found in the synapses and similarly to NgR1 it exists both pre- and post synaptically (Lee et al., 2008; Liu et al., 2003; Wang et al., 2002c).

THE NOGO-SYSTEM AND CNS INJURY

There has been a substantial amount of research examining the effects of the Nogo-system and how it can affect neuronal outgrowth after injury. Most of the initial research was on neuronal outgrowth following spinal cord injury. More recently, studies in stroke models have been carried out.

REGROWTH AFTER INJURY/TRAUMA

In studies examining the role of the Nogo-system in CNS injury, two different strategies have been used, based on the administration of inhibitors (around the time of damage) or the use of knock out animals (removing specific proteins, usually from birth). Several studies have shown increased neuronal outgrowth following spinal cord injury when blocking NgR1 function (Li and Strittmatter, 2003; Wang et al., 2006; Wang et al., 2011; Wang et al., 2012). However, even though neurons of the corticospinal tract (CST) express NgR1 it does not appear to regenerate better in NgR1-/- mice (Kim et al., 2004; Zheng et al., 2005) while raphespinal and rubrospinal fibers do (Kim et al., 2004). Enhanced growth was also seen in NgR1-/- animals in an ocular crush model (Wang et al., 2012), especially when combined with zymosan (macrophage activation). Blocking Lingo-1 (Ji et al., 2006; Lv et al., 2010), as well as the downstream effector molecules RhoA (Dergham et al., 2002; Fournier et al., 2003; Lord-Fontaine et al., 2008) and Rock (Duffy et al., 2009; Fournier et al., 2003) have also been shown to enhance outgrowth. There are several factors that influence neuronal outgrowth and an interesting example comes from the studies of Nogo knock-out animals; three different labs created three different Nogo knock-out mice that showed marked discrepancies in their regenerative phenotype (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003). Later studies have confirmed that effects such as age, strain and type of lesion affects the regenerative response (Cafferty et al., 2007a; Cafferty et al., 2007b; Dimou et al., 2006). When OMgp and MAG were also knocked out in a Nogo-A null background that still retains a small N-terminal part of Nogo-A, significant regeneration of corticospinal tract fibers was seen in a mouse model of spinal cord injury (Cafferty et al., 2010). In contrast, in mice with complete knock-out of Nogo-A, no regeneration of the CST was noted (Lee et al., 2010). Acute blocking of Nogo-A has been shown in several studies to be beneficial for outgrowth (reviewed in (Akbik et al., 2012; Gonzenbach and Schwab, 2008; Pernet and Schwab, 2012; Xie and Zheng, 2008).

Beneficial effects of blocking the nerve growth inhibitory systems have also been seen in stroke models (Cheatwood et al., 2008; Lee et al., 2004). Using viral mediated RNAi against NgR1 it was shown that the amount of contralateral fibers to the red nucleus and striatum increased after NgR1 silencing (Wang et al., 2010). In a second study (Shehadah et al., 2010), a combinatorial treatment of rats exposed to middle cerebral artery occlusion showed an increase in recovery and the treatment also resulted in a significant reduction in NgR1 expression. This fits well with the fact that outgrowth after injury is enhanced in slice culture after Nogo-A (Craveiro et al., 2008) or NgR1 inhibition (Mingorance et al., 2006). Another Nogo receptor, PirB, has been shown to be able to bind MAG, OMgp and Nogo and induce growth cone collapse in vitro (Atwal et al., 2008) as well as increase plasticity in the visual cortex (Syken et al.,

2006). However, lack of PirB has not led to improvements of regeneration following traumatic brain injury (Omoto et al., 2010) or spinal cord injury (Nakamura et al., 2011).

REGULATION OF MYELIN INHIBITORS

There is substantial evidence to support the notion that neurons are highly plastic in the intact brain and that sensory inputs can cause structural synaptic plasticity (Bhatt et al., 2009; Holtmaat and Svoboda, 2009; Yang et al., 2009). It is therefore likely that the normal inhibition of neurite growth in the CNS is modulated by neuronal activity to allow for local plasticity. In fact, forced neuronal activation by Kainic acid (Josephson et al., 2003; Karlen et al., 2009; Mingorance et al., 2004; Wills et al., 2012) results in a rapid downregulation of NgR1 in both hippocampus and the cerebral cortex (Josephson et al., 2003). In the same study, NgR1 was also shown to be downregulated in the brain in rats given access to running wheels (an activity that has been shown to increase synaptic plasticity). This downregulation could allow for increased plasticity at specific positions during a limited time window. Support of this hypothesis came when it was shown that NgR1 was downregulated in the somatosensory cortex in rats subjected to a thoracic spinal cord injury (Endo et al., 2007). The downregulation of NgR1 was specifically seen in those parts of the somatosensory cortex that responded to stimulation of the hind- and forelimbs. Using fMRI it was shown that the area that used to be activated by the forelimbs expanded into the area that used to receive input from the hind limbs; hence the downregulation of NgR1 was specific to the areas that were undergoing plastic changes. NgR1 is also downregulated at 7-15 days following hippocampal lesions (Mingorance et al., 2004). Of note, there was a small upregulation 24 h after the lesion. To conclude, NgR1 is downregulated in association with increases in neuronal activity or plastic changes. Following middle cerebral artery occlusion NgR1 expression was shown to increase (Wang et al., 2010). However, when rats with middle cerebral artery occlusion received a combinatorial treatment that resulted in functional improvement, NgR1 levels were decreased suggesting that lower than normal levels of NgR1 expression can be beneficial for neuronal outgrowth/repair (Shehadah et al., 2010). While Nogo-A levels generally appear to be more stable increases have been noted in a rat stroke model in cortical areas after 28 hours (Cheatwood et al., 2008). Interestingly, there was a transient downregulation during the first 1-14 days after the stroke (Cheatwood et al., 2008).

NOGO-RECEPTORS AS REGULATORS OF SYNAPTIC PLASTICITY IN THE INTACT CNS

Recently, there has been an increased interest in the possible roles of MAIs in the uninjured nervous system. Given that NgR1 is expressed selectively by neurons, and that Nogo is also expressed by a set of neurons, one focus of interest has become gray matter and structural synaptic plasticity.

MYELIN ASSOCIATED INHIBITORS IN EXPERIENCE-DEPENDENT PLASTICITY

The first conclusive evidence that the Nogo system is involved as a regulator of plastic changes came in studies on plasticity in the visual cortex after monocular deprivation. It has long been know that by closing the contralateral eye, the visual cortex will increasingly start to respond to information from the ipsilateral eye (Hofer et al., 2006). In a paper from 2005 McGee and colleagues found that plasticity in the visual cortex remained high after monocular deprivation past the critical period for such plasticity (that normally ends around day 32), in mice that lacked NgR1 (NgR1-/-) (McGee et al., 2005). In fact even NgR1-/-mice that were 4 months old exhibited significant plasticity in their visual cortex. Furthermore, when Nogo-A/B was knocked out plasticity was increased similarly as seen in mice lacking NgR1, suggesting that Nogo signaling through NgR1 could be a mechanism by which plasticity is normally suppressed following closure of the critical period. Nogo-A, OMgp and MAG can also bind to a second receptor called PirB (Atwal et al., 2008) and deletion of this receptor has also been shown to result in increased plasticity in the visual cortex following monocular deprivation (Syken et al., 2006). Furthermore, rearing mice in the dark has been shown to delay the maturation of GABAergic neurons as well as the deposition of CSPG into perineuronal nets and affect the monocular response (Pizzorusso et al., 2002). Recently it has also been shown that NgR1 as well as NgR3 works as receptors for CSPGs suggesting that NgR1 might also play a role in the CSPG induced response as well (Dickendesher et al., 2012).

STRUCTURAL PLASTICITY

The first evidence supporting a role for the Nogo-system in plastic responses in the uninjured CNS came when it was shown that addition of IN-1 antibody to uninjured cerebella resulted in increased axonal sprouting already after two days, an effect that disappeared after one month (Buffo et al., 2000). When organotypic hippocampal cultures were treated with an anti-Nogo antibody it resulted in significant changes in dendritic structures as measured by Scholl analysis. When NgR1 or Nogo-A were inhibited with a blocking antibody there was a profound effect dendritic structure and spine types, while changes after Nogo-A deletion were smaller (Zagrebelsky et al., 2010). Nogo-A knock-out mice have also been shown to have increased growth cone motility and this appears to be dependent on changes in RhoA activation (Montani et al., 2009). It has recently been shown in both cell and tissue culture that loss of NgR1, NgR2 or NgR3 independently will result in an increase in spine density (Wills et al., 2012). Furthermore, overexpression of NgR1 resulted in decreased spine density in vitro. *In vivo*, a single knock-out of any of the three NgR genes did not result in an increase in spine density. However, when all three Nogo-receptors were knocked out, a

significant increase in spine density was seen *in vivo* (Wills et al., 2012*)*. It has also been shown that this effect appears to be mediated by the co-receptor TROY and the downstream regulator RhoA. It was also confirmed that NgR1 is regulated by neuronal activity as previously demonstrated (Josephson et al., 2003) but also that NgR2, NgR3 and TROY are regulated by neuronal activity.

ELECTROPHYSIOLOGICAL PLASTICITY

Changes in electrophysiological activity such as LTP and LTD have been linked to changes in synaptic structure (Saneyoshi et al., 2010). Addition of Nogo-66 or OMgp to hippocampal slices result in reduced LTP in a NgR1 dependent manner (Raiker et al., 2010) and blocking Nogo-A or NgR1 using blocking antibodies increase LTP (Delekate et al., 2011). While NgR1-/- mice do not show any impairment in LTP induction (Lee et al., 2008; Raiker et al., 2010) they do exhibit impairment of LTD. When NgR1 is instead overexpressed, the formation, length and de-potentiation of LTP is similar to that seen in control mice (Karlen et al., 2009), even though such overexpression severely impairs the ability to from lasting memories (see results below).

MYELIN INHIBITORS IN DISEASE

The Nogo-system has been implicated in both neurodegenerative and neuropsychiatric diseases. For instance, it has been shown that Reticulon members (He et al., 2004), one of them being Nogo-A, can bind to and modulate the activity of BACE1, a key molecule in the formation of Aβ (Hardy and Selkoe, 2002). Nogo knockout mice that overexpressed APP showed less impairment in the Morris water maze compared to control mice, without affecting plaque load (Masliah et al., 2010). The expression of synaptic proteins was however normalized, and a possible conclusion is that loss of Nogo-A can partly rescue the memory impairment in these mice by increasing compensatory plasticity. Nogo-B expression has been shown to increase in aged and Aβ treated rats, while Nogo-A expression was not changed. Nogo-B expression was correlated with increased activity of microglia and impairments of LTP induction (Murphy et al., 2011). Of note, Nogo-A expression has been shown to increase in a group of aged rats with cognitive impairment compared to age matched controls and the Nogo-A levels had an inverse correlation with performance (VanGuilder et al., 2011).

Nogo receptors have been implicated in modulating plaque formation in a mouse model of Alzheimer's disease (APPswe/PSEN1). Park and colleagues showed that NgR1 can bind to APP and Aβ and that NgR1 overexpression in neuroblastoma cells decreased Aβ production (Park et al., 2006a). When NgR1-/- mice were crossed with APPswe/PSEN1 mice, NgR1-/-/APPswe/PSEN1 animals had a higher plaque load than NgR1+/-/APPswe/PSEN1 mice, suggesting that NgR1 can influence plaque formation. In a follow up study, subcutaneously delivered soluble NgR1 decreased plaque loads (possibly by working as a peripheral sink for Aβ) and attenuate the age related decline in cognitive function seen in APPswe/PSEN mice with control treatment (Park et al., 2006b). There are also reports to suggest that while the neuronal density in Alzheimer patients decrease, the density of NgR1 expressing neurons stays rather stable in the dentate gyrus (Zhu et al., 2007), suggesting that NgR1 might have some protective role or that neurons that do not normally express NgR1, induce expression in Alzheimer

patients. Zhou and colleagues (Zhou et al., 2011) confirmed that NgR1 can bind to APP but unlike the study by Park and colleagues (Park et al., 2006a) they found an interaction between APP and all NgRs, but no interaction between NgRs and Aβ. Zhou and colleagues also found that overexpression of NgR2 (and to a lesser extent NgR1 and NgR3) increased the formation of Aβ and mice lacking NgR2 had reduced depositions of Aβ.

The Nogo system has also been linked to schizophrenia, and mutations in NgR1 (that affect Nogo binding) have been found in patients with schizophrenia. NgR1-/- mice show some phenotypes akin to schizophrenia (Budel et al., 2008). Mice lacking Nogo-A were also found to exhibit phenotypes common in schizophrenia (Willi et al., 2009; Willi et al., 2010).

MEMORY

The brain has the capability of storing our experiences as long-term memories that in some instances can be recalled for the rest of our lifetime. The form in which these memories are stored has been difficult to elucidate. However, there is now a growing agreement that long-term memory formation involves structural changes in the neuropil. The formation of new contacts and the elimination of old contacts cause an alteration of the synaptic network which in turn carries the new memory.

As plastic changes within telencephalon are believed to be the anatomical foundations of memories, it is interesting that the CNS of mammals is highly inhibitory to neuronal outgrowth. The aim of this thesis was to investigate how some of these inhibitors (mainly the NgR1) are regulated by neuronal activity and to see if they can influence the formation of long-term memories.

Memory does not appear to exist in a single functional system that handles all types of information that is intended to be retained. Current models of memory instead describe a variety of different memory systems that can work, to some extent, independently so that damage to one system will not necessarily impair function in other systems. These memory systems do not only differ in the type of information that they handle but they also appear to utilize different regions of the brain for their function.

WORKING MEMORY

The terms short term memory and working memory are sometimes used as synonyms and sometimes with distinction, generally that working memory involves conscious manipulation of information while short term memory typically refers to storing it (Baddeley, 2003). In this thesis they will both be described as "working memory" and refer to information that can be held in mind simultaneously without the involvement of long-term memory. Some studies suggest that when working memory is stretched to its limit it might also engage the medial temporal lobe/hippocampus (Jeneson and Squire, 2012; Squire and Wixted, 2011).

DESCRIPTIVE MEMORY

Descriptive memories are the foundation of what is normally considered our long-term memory and are divided into semantic memories (facts) and episodic memories (experiences). These memories can either be retained for a brief interval (for example, when cramming for an exam) or can be long-term as (hopefully) remembering your door/pin code. The exact relation between semantic and episodic memory is not established but it has been postulated that semantic memory forms a foundation that higher order memories (episodic) can be built upon (Eustache and Desgranges, 2008). There are suggestions that semantic and episodic memories uses somewhat different brain systems (Vargha-Khadem et al., 1997), while other studies support the claim that hippocampus is essential for both types of memories (Manns et al., 2003a; Manns et al., 2003b).

Long-term memory refers to information that can be stored for a prolonged period of time in the brain. A key to our understanding of which brain structures that are needed

for the formation of long term memories were the observations of a neurosurgical patient (H.M.) who in 1953 (Scoville and Milner, 1957; Squire, 2009) had surgery to remove parts of his medial temporal lobes bilaterally, to address irretractable epileptic seizures. What was not known at the time, but became apparent after, is that the medial temporal lobes are essential for the formation of new long-term memories. H.M. was not impaired on IQ-tests following the surgery (in fact his performance improved) but his ability to form new descriptive memories was severely impaired. The lesion in H.M. was far too large to specifically identify the important regions but following the discovery of the importance of the medial temporal lobe in memory formation, several other reports both in patients (Manns et al., 2003a; Manns et al., 2003b; Rempel-Clower et al., 1996; Zola-Morgan et al., 1986) and in animals (Squire and Zola-Morgan, 1991; Zola-Morgan et al., 1986; Zola-Morgan et al., 1994) have clarified the structures involved in long term memory formation. The general picture that has emerged is that while damage to the hippocampus (for instance only the CA1 area) was sufficient to result in memory impairment it is not enough to cause an impairment that is similar in extent to that seen in H.M. Instead it appears that other structures such as the entorhinal, perirhinal, and parahippocampal cortices are also involved in memory processing and together they make up a large part of the parahippocampal gyrus. MRI studies of patients that exhibit memory impairments after anoxic experiences have repeatedly shown reduction in hippocampal volume of around 40% (Cipolotti et al., 2001; Isaacs et al., 2003; Shrager et al., 2008; Squire et al., 1990). Regions outside of the telencephalon, most notably in the diencephalon, are also important for memory functions and several studies have implicated nuclei in thalamus (especially the medial dorsal nucleus) in memory impairments (Gold and Squire, 2006; Harding et al., 2000; Mayes et al., 1988; Squire et al., 1989).

A very important distinction is that while the medial temporal lobes are required for the formation of long-term descriptive memories, it does not appear to be the storage site for these memories. Instead it is believed that long-term memories reside in neocortex (Danker and Anderson, 2010) close to the regions that originally interpreted the information and that have been shown to be re-activated during retrieval (Nyberg et al., 2000). Hence damage to the region of the brain that process color would impair retrieval of "colorful memories", something that has also been seen in a patient (Sacks, 1995). The amnesia seen after medial temporal lobe lesions is generally antereograde, but retrograde amnesia of a period ranging from a few years up to a decade before medial temporal lobe damage can be seen depending on the extent of the lesion (Bayley et al., 2006; Bright et al., 2006; Manns et al., 2003b). In this thesis memories that last for longer than three weeks will be defined as lasting memories and as will be described in the results section these type of lasting memories are affected by overexpression of NgR1.

NON-DECLARATIVE MEMORY

These are memories that are stored in the brain and influence our behavior but unlike declarative memories, we are not aware of how the information contained in non-declarative memories is stored. For example we know how to ride a bike but cannot explain how we specifically learned to master it.

Perceptual memories

Perceptual memories refers to information that is perceived and that can later affect our behavior but without entering our conscious mind. For instance when people are shown words in one context such as counting the number of letters in each word the brain will unconsciously be primed for these words. When subjects are later shown only the first few letters of these words and are asked to fill in the last part, they use the words shown earlier at a higher frequency then other words starting with the same stem, even though they have no conscious recollection of previously seeing the words (Tulving, 1982). Patients with severe MTL lesions have been shown to have normal perceptual memories but these may not be as long lasting as in controls (Squire et al., 1987).

Procedural memories

Procedural memory is generally considered distinct from other types of memories discussed above, and has received substantial attention as a system underlying many of our acquired motor skills. For instance while H.M. was severely impaired with regard to descriptive memories, his performance was comparable to controls in learning the mirror drawing test. Using a group of amnesic patients it was shown that they could learn to read mirrored words as well as controls (Cohen and Squire, 1980). Amnesic patients when confronted with tasks that cannot be learned, (outcome is probabilistic and relies on "gut feeling"), perform equally well as controls (Knowlton et al., 1996). Amnesic patients could learn an eight-pair discrimination task using habit memory, but instead of the 80 trials that the controls required, amnesic patients required > 1000 trials and were not aware of the fact that they had practiced the test (Bayley et al., 2005). Rodents with hippocampal lesions can still learn a version of the Morris water maze when the starting location is held constant (always same path to platform) but are deficient when the start location varies (require the use of external cues to locate the platform. Procedural memories appear to require both striatum and cerebellum and patients with Parkinson's disease show impairments in tests involving procedural memory (Knowlton et al., 1996).

SYNAPTIC PLASTICITY

For more than a century it has been believed that both spines and the structure of dendrites are important for memory storage (Garcia-Lopez et al., 2007; Shepherd and Erulkar, 1997). Raisman provided convincing evidence for synaptic alterations in the brain at the EM level by deafferentation studies in the septal nucleus (Raisman, 1969) and a similar occurrence of denervation-induced alterations in the hippocampal formation was later seen (Cotman et al., 1973). It has also been shown that spines are highly dynamic structures. For instance, a synapse loss of around 50% is seen during the postnatal period (Huttenlocher, 1979; Rakic et al., 1986; Rakic et al., 1994). After this major change, the synaptic densities remain rather stable through to adulthood until a small decline can be seen in aged individuals (Duan et al., 2003; Terry et al., 1991). If spines are indeed part of the neuronal substrate for memory, it would be necessary for spines (at least some of them) to also be highly stable. While there are some contradictory results regarding spine stability, the overall picture suggests that there are indeed spines that are stable for very long time periods and that the difference that was initially seen was due to different methods and did not reflect differences in factual spine dynamics (Holtmaat et al., 2005; Mizrahi et al., 2004; Pan and Gan, 2008; Xu et al., 2007). If spines are indeed important for the formation of memories then they

should change in response to stimuli. When LTP is induced in brain slices there is also an increase in dendritic growth and spine formation (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999; Zhou et al., 2004) while LTD induction reduces spine numbers and results in spine shrinkage (Nagerl et al., 2004; Zhou et al., 2004). Furthermore, increases in spine density have been reported after environmental enrichment, rearing training and long term sensory stimulation (Greenough et al., 1985; Knott et al., 2002; Kolb et al., 2008; Moser et al., 1994). In a highly influential paper it was shown that formation of new spines correlated with improvements in the accelerating Rotarod and that some of the newly formed spines were highly stable (months) (Yang et al., 2009). Spine density can also be increased by the administration of drugs of abuse; interestingly while both psychostimulants and opiates show very similar initial behavioral responses the changes in neuronal structures are opposite (Luscher and Malenka, 2011; Russo et al., 2010). Thus psychostimulants increase spine density while opiates have the opposite effect. There are several proteins that influence spine dynamics (Bhatt et al., 2009) but of specific interest for this thesis is the notion that RhoA is highly involved (Linseman and Loucks, 2008; Schubert and Dotti, 2007).

PLASTICITY IN HUMANS

While the possibilities to study spine dynamics in animal models has increased tremendously by the development of *in vivo* two-photon microscopy, the possibilities to study spine changes and dynamics in humans is still challenging at best. Most studies have focused on indirect methods such as change in size of brain areas. One of the first studies to implicate changes in brain structure with activity in humans was the a study showing that taxi drivers in London had larger posterior hippocampi (but smaller anterior hippocampi) than control individuals (Maguire et al., 2000). Following this study, several studies have shown changes in brain structure as the result of a plethora of circumstances; for instance in students studying for an extensive medical exam both the hippocampus and part of the parietal lobe increased in size (Draganski et al., 2006). Many other examples exist and include several different types of experiences (reviewed in (Fields, 2011). However, most of these studies have used MRI or similar techniques so it is not possible to know the exact substrate of this plasticity. For instance, it is appealing to assume that training would result in an increase in dendritic arborization and an increase in spine density but other explanations such as increased vascularization cannot be ruled out.

AIMS

To investigate the distribution and regulation of the Nogo-receptors and the NgR1 inhibitor LOTUS

To analyze the effect of NgR1 overexpression on lasting memories

To determine if NgR1 overexpression in forebrain neurons affects plaque deposition or cognition in a mouse model of Alzheimer's disease

To examine if NgR1 overexpression affects the formation and stability of a behavioral sensitization induced by psychostimulants

To analyze if NgR1 overexpression affects spine dynamics

MATERIALS AND METHODS

ANIMALS

Experiments in this thesis were approved by the Stockholm Animal Ethics committee.

MemoFlex mice

NgR1 overexpressing mice (MemoFlex) were created by cloning a 1.4 kb fragment of mouse NgR1 into the HindIII-XbaI site of pTRE2 mice (BD Biosciences Clontech). The resulting plasmid was purified and microinjected into pronuclei of fertilized mouse eggs from C57BL/6 mice (MouseCamp, Karolinska Institutet). The resulting mice, pTRE-NgR1 were crossbred with mice carrying a tTA transgene under the control of the CamKII promoter (Jackson Laboratories) to create MemoFlex mice. The overexpression of NgR1 can be turned off by adding doxycycline to the drinking water (100 mg /l in paper I and 200 mg/l in paper III).

NgR1 knockout mice

The NgR1 knockout mice used in study III were a kind gift from Dr. Marc Tessier-Lavigne (Genentech,USA) and have been described previously (Zheng et al., 2005).

Housing

Mice were group housed separated based on sex (weaned at 3 weeks of age) in cages with access to food and water *ad libitum.* Mice were kept on a 12/12 hour light/dark cycle; lights were on between 06:00 and 18:00. The temperature was kept at 22-23°C with a relative humidity of 60 %. In the cages, mice had access to a small paper house and tissue paper as "enrichment".

LOCOMOTOR, BALANCE AND COORDINATION TESTS

Rotarod

To assess if overexpression of NgR1 would result in impairment of motor function we analyzed the performance of mice in a Rotarod test. Mice are put on a horizontal rod that can change rotating speed and the time they stay on the Rotarod is measured. During the first day mice were exposed to three training sessions. In the first, mice were habituated with a stationary rod and in the two subsequent trials the rod rotated at a fixed speed (4 rpm). The trials were 180 s with a 60 s inter-trial rest period. On the second day the rod was set to accelerate from 4-40 rpm and each mouse was trained twice for 180 s (60 between trials). On the third day (test day) two trials were administered with an accelerating rod (4-40 rpm) with a trial length of 300 s (60 s between trials). The latency to fall (or rotate off the top of the rod) was measured by the Rotarod timer and the average for the two trials was used as a measure of locomotor proficiency for the mouse.

Locomotion and Open Field

We used a multi-cage automated system that uses infrared beams to measure locomotor activity and explorative behavior (AccuScan VersaMax, Accuscan Instruments) to assess the amount of basal locomotor activity in MemoFlex mice. This apparatus

consists of eight transparent Plexiglas chambers (42 * 42 *30 cm) connected to a computer system that measures the number of beam breaks. These are then converted to horizontal and rearing activity and summed for 5 min bins for 60 min total.

Elevated Plus-Maze

To assess anxiety associated behavior the elevated plus maze system was used. This maze consists of 4 arms (30 cm long 5 cm wide); two of these are closed with gray walls and two of the arms are open (lacking walls). The maze was elevated 60 cm from the ground and the amount of time and entries to the closed and open arms are recorded using a camera-based system (Ethovision, Noldus).

Running Wheel

Mice that are given free access to running wheel will gradually increase the distance they run in the wheels. MemoFlex mice and control mice were given free access to running wheels. During the experiment mice were single-housed in cages (22*16*14 cm) with running wheels (r=12.4 cm; one revolution = 39 cm) for 5 weeks and the running activity was measured continuously. During the experiment the animals had free access to both food and water.

MEMORY TESTS

Morris water maze

Morris water Maze (MWM) (Morris, 1984) is one of the most widely used tests for testing visuospatial memory (Vorhees and Williams, 2006) in mice and has been shown to rely heavily on the function of the hippocampus (D'Hooge and De Deyn, 2001; Sharma et al., 2010).

We used the Morris water maze in papers I and II to assess the ability of our different mice strains to form spatial hippocampal dependent memories. In our setup of the MWM we use a circular tank (180 cm in diameter) with a hidden platform (10 or 15 cm) in diameter. Surrounding the tank several external cues were placed to assist the mice in "triangulating" the location of the platform. North in the tank was defined as the pool position furthest from the experimenter and south as the closest, west is to the left of the experimenter and east to the right. These positions were used as starting positions for the mice. Mice were trained for 4 trials per day during 7 days (28 trials in total). In each trial the mice were semi-randomly (never the same starting location twice in one day) put into one of the four starting points (north, east, south and west). There was at least 30 minutes between two trials for the individual mice. During the learning phase the platform remained in a constant position in one of the four quadrants of the water maze. We used the Morris water maze to assess hippocampal dependent memory. To verify that the learning was indeed hippocampal dependent and not due to other strategies used by the mice, such as swimming at a constant distance from the wall, we used probe trials (without platform) at various time-points (after the learning phase or during follow-up tests of long lasting memory) to assess if mice spend more time in the quadrant that used to contain the platform compared to the 3 other quadrants. After putting mice into the tank the experimenter moves to an adjacent room and the performance of the mice is recorded using a video system and several

parameters (latency, distance, swim speed, thigmotaxis etc) can be analyzed using a computer program (Water Maze Software). To assess how stable the learning of the platform location had become in our mice we retested them in the MWM after a prolonged delay (39-60 days).

Radial Arm Water maze

In paper II mice were also tested in a radial arm water maze (RAWM) that compared to the open pool in MWM instead consists of a small open center zone (40 cm in diameter) from where 6 arms (30 cm long and 19 cm wide) emanate. In one of the 6 arms a hidden platform was placed and the other 5 arms were used as starting locations. The mice were trained in the RAWM for 4 days with 4 trials per day. During each trial mice were semi-randomly (never same arm twice) put into one of the empty (no platform) arms and during 60 s the number of entries into arms that did not contain a platform was recorded. The location of the platform was held constant during the learning phase but changed between experiments (some mice were tested in the maze more than once).

Passive avoidance

Memories that involve aversive events (fear conditioning) are generally thought to rely heavily on the function of amygdala. (LeDoux, 2007; Sigurdsson et al., 2007). One way to assess fear memory is to use a contextual system where in a two compartment box one room is associated with fear while the other room is not. The preference for the non-associated box can be used to assess the strength of the fear memory.

We used two different systems (Ugo Basile and TSE Systems) that differed somewhat in how movement of mice was measured and in the size of the boxes to test passive avoidance (paper I). The learning phase for these tests consists of a single exposure; mice were put into the bright side of a two compartment box with one illuminated (bright) side and one dark side. The two sides of the box are separated by a door that is closed when the mice is put into the box. After 60 s of exploration the door will open and mice will have access to the dark compartment as well. When the mouse enters the dark compartment the door will close and a single foot shock will be administrated (0.3 mA, 2 s, via a metal grid floor). Thereafter, the mouse was left in the dark compartment for 30 s after the shock before being removed (a maximal time of 300 s is set and if mice did not enter they were removed from the study). To investigate the retention of these memories, mice were re-exposed to the apparatus after 1 and 7 days as well as after 4 weeks. During these retention tests the door between the compartments was left open during the entire trial time (300 s) and no shock was administered and the time before mice entered the dark compartment was recorded (mice that did not enter the dark compartment were assigned the time 300 s).

Drug induced sensitization

It is well known that mice that are given psychostimulants will show an increase in locomotion with repeated administration, even if the dose is held constant. In paper III we exposed both NgR1 overexpressing mice and NgR1 knockout mice (NgR1-/-) to a sensitization paradigm.

To measure drug induced locomotor activity, mice were put into AccuScan boxes as previously described. This enabled us to closely measure locomotor activity and also provides a new environment for the sensitization, something that has been shown to enhance the sensitization response (Badiani et al., 1995). We used a 9 day sensitization paradigm, during the first two days mice get saline injections and for the last 7 days they will receive injections of D-amphetamine 2mg/kg (Lipomed, Arlesheim, Switzerland), both I.P. All trials were 90 min long and after the experiment mice were returned to their home cages. The Accuscan boxes also recorded stereotypy behavior.

CELLULAR EXPRESSION OF MRNA SPECIES

Oligonucleotide probes

One way to measure mRNA levels is to use quantitative *in situ* hybridization (ISH). It has been shown that expression levels measured by ISH and quantitative PCR correlate strongly (Broide et al., 2004), confirming accuracy of quantitative ISH. In papers I and IV ISH using oligonucleotide probes of ~50 bp were used to analyze the expression of mRNAs of interest. To ensure that the probes were specific they were first aligned to all publicly known sequences using UCSC genome browser (http://genome.ucsc.edu/) and folding energy was assed using Mfold (Version 3.2) (Mathews et al., 1999; Zuker, 2003). Two or more oligonucleotides, targeting different parts of a given mRNA of interest were created and the expression of both were compared before one was chosen for the experiments (expression patterns were identical but some probes generated stronger signals and were chosen for subsequent experiments), expression patterns were also compared to previously published data when available. The following oligonucleotides were used:

Paper I Nogo-A: (5 -GCT CTG GAG CTG TCC TTC ACA GGT TCT GGG GTA CTG GGG AAA GAA GCA-3), NgR1: (5 –AGT GCA GCC ACA GGA TGG TGA GTA TCC GGC ATG ACT GGA AGC TGG C-3), transgene-specific NgR1: (5 -GGA GGC TGG ATC GGT CCC GGT GTC TTC TAT GGA GGT CAA AAC AGC GTC-3), endogenous-specific NgR1: (5 -TTC GGG GTC GAG CGG GGC GCG TCG GGC ACT GGA AGC GGC TTC GGG GCG-3), Lingo-1: (5 -TCC AAG ACC TTG AGT CGG TAC AGC CTC TTG AAG GAG TAG TCC CGG ATG GC-3), Troy: (5 -TTT ATT CCT GCT ACT CGC CAG TGC TGT GCT CCA GAC TCA CGC TTT CCG-3), p75 (5 –GGC CAC AAG GCC CAC GAC CAC AGC AGC CAG GAT GGA GCA ATA GAC AGG-3) and BDNF (5 -CTC CAG AGT CCC ATG GGT CCG CAC ACC TGG GTA GGC CAA GCT GCC TTG-3)

Paper IV: NgR1: (5-GTG CAG CCA CAG GAT AGT GAG ATT TCG GCA TGA CTG GAA GCT CGC AGC TTC GGG GCG-), NgR2: (5-AGG GCG CTC AGT CCA CAC TTA TAG AGG TAG AGG GCG TGA AGC TTC-3), NgR3: (5-AAG GAC AGC GGC ACT GAG GAG AAG TTG TTG GCC TGG CAG CTC ACG GT-3)", LOTUS: (5-ACA GAC AGT GGC TGA GCC ATG GAC TCT CCA TGT GAC AAG ATG AGA TAA AGC A-3).

In situ hybridization

In situ hybridization was performed based on the procedure developed by Dagerlind and colleagues (Dagerlind et al., 1992). Probes were labeled with ^{33}P at the 3' end using terminal deoxynucleotidyl transferase (TdT) (Amersham Biosciences, Buckinghamshire, UK) and purified using ProbeQuant G50-Micro columns (Amersham Biosciences). Before processing, cryosections were air-dried for 3-5 h after which they were hybridized over night at 42°C in a humidified chamber; labeled Oligonucleotides were added to the hybridization mixture and covered with Parafilm (Parafilm M, VWR international). After overnight incubation slides were washed 5 times in 1X SSC buffer (60°C) for a total time of an hour, cooled to room temperature and dehydrated using rapid submersion in increasing concentrations of ethanol (50%, 95%, 99.5%) and air dried.

Visualisation of probe radioactivity

When the ISH slides have dried they are placed overnight with phosphoimaging plates to ensure quality and strength of labeling and to determine the exposure time needed for film autoradiography (Fujix BAS-3000; Fuji Photo Film Co, Tokyo, Japan). When an appropriate time exposure time has been established, slides were placed on autoradiographic films (Biomax; Eastman Kodak Co, Rochester, NY, USA), a ^{14}C standard was also included and the slides were left on film for between 2 days and 4 weeks, depending on the strength of the probe. The films were developed and scanned (Epson perfection pro V750, Epson, Nagano, Japan) and the expression in defined areas was quantified using appropriate software (ImageJ (V1.32 and 1.44p), http://rsb.info.nih.gov/ij/). Measured expression levels are then converted to nCi/g (Paper I) or expressed as percent of controls (Paper IV).

Western blot

For paper I, adult mouse tissues (hippocampus, olfactory bulb, spinal cord, cortex and striatum) were dissected and frozen using liquid nitrogen and kept in a -80°C freezer until used. Tissues were sonicated in 1% sodium dodecyl sulfate and boiled for 10 min. Aliquots of the homogenate were used for protein content determination using the bicinchoninic acid protein assay method (Pierce). Protein (60 µg) from each sample were loaded onto a 10% polyacrylamide gel and separated by SDS-polyacrylamide gel electrophoresis after which the proteins were transferred to polyvinylidine difluoride membranes (Amersham Pharmacia Biotech). Membranes were next immunoblotted using a Goat anti-NgR1 antibody (R&D Systems). Anti-body binding was revealed by the secondary anti-goat IgG antibody (Rockland) and the Odyssey System immunoblotting detection system.

In a second experiment, NgR1 protein expression was analyzed after adding doxycycline. Fresh frozen mouse tissues were lysed and sonicated in 0.5% Triton X-100, 3% SDS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA containing protease inhibitor mixture (Sigma), NaF, and phosphatase inhibitor cocktails 1 and 2 (Sigma). The samples were added to SDS/PAGE (NuPage 4–12% Bis-Tris Gels, Invitrogen) followed by immunoblotting. Membranes were incubated with primary antibodies against NgR1 (R&D Systems), GAPDH (Abcam), and N-Cadherin (Novus Biological) washed and then probed with a secondary antibody (Alexa-680, Invitrogen

or IRDye-800, Licor). The membranes were later scanned using an infrared scanner (Odyssey, Licor).

Membrane fractionation

Fresh frozen mouse tissues were homogenized using a Dounce homogenizer in hypotonic buffer (10 mM NaHCO3 with protease inhibitor and phosphatase inhibitors cocktails). After 10-min incubation on ice, nuclei and cell debris were removed by centrifugation at 1 200 * g for 10 min. The resulting supernatant was removed and centrifuged again, this time at 21 600 * g for 30 min in a Beckman L7 ultracentrifuge to remove the internal membranes. This was followed by centrifugation at 150 000 * g for 2 h to isolate plasma membranes. The pellet containing the plasma membrane was washed by resuspension in hypotonic buffer and centrifuged at 150 000 * g for 2 h and then solubilized in an extraction buffer containing 0.50% Triton-X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% SDS, and protease inhibitor and phosphatase inhibitors cocktails. The expression level of NgR1 in the plasma membrane fraction was determined by SDS/PAGE (NuPage 3–8% Tris-acetate Gels, Invitrogen) followed by Western blotting as described above.

RhoA activity assays

For paper I, a commercially available ELISA-based RhoA activity assay (G-LISA; Cytoskeleton) was used to measure the relative RhoA activity of hippocampus from L1 and control mice.

Immunohistochemistry

Mice were deeply anaesthetized with pentobarbital and a needle was inserted into the heart through which first 10 ml of Thyrode and then 50 ml of fixation solution (4% formaldehyde with 0.4% picric acid in PBS) were perfused. The brains were removed and immersed in the same fixative; later they were rinsed and kept in sucrose (changed >3 times). Brains were later cryosectioned (Microm HM 500 M, GM Inc USA, 14μm) and processed for immunhistochemistry. Sections were rehydrated in PBS for 10 min and covered with blocking solution (PBS with 0.1% Triton-x, 5% goat serum, and 2% BSA) for 1 h. After blocking, primary antibodies were added to the sections in a solution similar to the blocking solution but lacking BSA. For paper I, Goat anti-NgR1 (R%D systems) with an anti-goat Cy3 conjugated antibody as a secondary antibody was used. To label plaques in paper IV an anti Aβ-42 antibody (Cell signaling Danvers, MA, USA) was used together with a secondary anti-rabbit biotin conjugated antibody, visualized by an avidin HRP procedure (ABC-kit, Vector Laboratories Burlingame, CA, USA). To visualize NgR immunoreactivity, a goat anti-NgR primary antibody (R&D systems, MN, USA) and, as a neuronal marker, an anti-NeuN antibody (Millipore, MA, USA) was utilized. Signals were visualized using fluorescent secondary antibodies, anti-goat Alexa 488 and anti-mouse Alexa 535 (Invitrogen, NY. USA) were used. Fluorescent images were taking using a Zeiss LSM 510 Meta microscope and all other pictures were taken using Zeiss Axiophot.

Plaque quantification

To enhance the contrast of plaques compared to background, hue and saturation was modified using an automated script (same settings for all brain regions) and appropriate software (Adobe Photoshop®). The images were converted to black and white and plaque load (area occupied by plaques and plaque numbers determined using appropriate software (Imagej (v 1.44p, http://rsb.info.nih.gov/ij/). Identical settings

were used for all slides within a specific region. Serial sections were taken (every 10 section) and the average of 4-5 were analyzed and the average number was assigned as the value for a given mouse brain.

Golgi staining and spine counts

To visualize the dendritic tree of neurons and enable analysis of spine structure we used the Golgi method; this method randomly and completely stains a few neurons and leaves the majority of cells unstained. It is then possible to count individual spines on the scattered stained neurons without much interference from surrounding neurons. We used a rapid Golgi staining kit (FD Rapid Golgi kit, MTR Scientific) according to the manufacturer's protocol. Once the tissue was stained it was cryosectioned at 180 µm and spines were counted. For paper I we analyzed spine density on the apical dendrite of pyramidal neurons in cerebral cortex (at least 50 µm from the soma), for a distance of 10 µm. Spines were visually categorized as mushroom shaped or non-mushroom shaped. For paper III the same staining procedure was used but sections were performed using a microtome instead of a cryostat (200µm). Spines were measured on both distal apical dendrites on pyramidal neurons in the cingulated gyrus and on 2:nd and 3:rd order dendrites on medium spiny neurons in the shell of nucleus accumbens using dedicated software (Neurolucida, MicroBrightField inc, VT, USA). Spines were categorized as thin (thin and filopodia), mushroom or other.

High-performance liquid chromatography

Concentrations of monoamines (paper I) and their metabolites in brain tissue samples were determined by high-performance liquid chromatography (Andersson et al., 1995). The separations were accomplished using a reverse-phase column (Reprosil-Pur, C18-AQ). Monoamines and metabolites were detected using an electrochemical detector system with a high sensitivity analytical cell and appropriate software (ESA Coulochem III and EZ Chrom Elite; ESA, Dalco Chromtech AB). Tissue level values were expressed as ng/g wet weight.

Electrophysiology

Brains were removed and immersed in cold (4 °C), oxygenated media, composition (mM): NaCl, 87; KCl, 2.5; MgCl2, 7; CaCl2, 0.5; NaH2PO4, 1.25; glucose, 25; sucrose 45; and NaHCO3, 25. Transverse slices (280 m) were made using a vibrating tissue slicer (VT1000S, Leica Instruments), and incubated in normal media consisting of (mM): NaCl, 126; KCl, 3.0; MgCl2, 1.0; CaCl2, 2.4; NaH2PO4, 1.2; glucose, 11.0; and NaHCO3, 25, saturated with 95% O2 and 5% CO2. Individual brain slices were then moved to a recording chamber that was continuously perfused with normal media (3 mL/min), and maintained at a temperature of 30–32 °C. A blinded experimenter performed recordings in the CA1 region of hippocampus. Extracellular fEPSPs were recorded in stratum radiatum using glass micropipette electrodes filled with 3M NaCl, and an AC amplifier (A-M Systems Model 1800). The signals were high- (10 Hz) and low-pass (10 kHz) filtered, and acquired to a personal computer at 4 kHz via an A/D board (National Instruments PCI 6251), using appropriate software (WinLTP v0.95b, courtesy of Dr. William A. Anderson, University of Bristol, Bristol, U.K.). fEPSPs were elicited by electrical stimulation of stratum radiatum at a frequency of 0.033 Hz using single, 0.1-ms pulses, delivered through a bipolar electrode constructed using formvar insulated nichrome wire. After obtaining an input-output relationship (stimulus

intensity versus peak fEPSP amplitude) for each response, the stimulus intensity was adjusted to produce a baseline fEPSP with a peak amplitude of 0.5–1 mV (30–40% of the maximum response). After at least 10 min of stable baseline, LTP was induced by either (1) high frequency stimulation (HFS) consisting of three trains of 1-s duration at 100 Hz, delivered at 20-s intervals; or (2) theta-burst stimulation (TBS) consisting of 10 bursts of five pulses at 100 Hz, with a 200-ms inter-burst interval. Both HFS and TBS were delivered at the stimulus intensity used to elicit the baseline responses. Peak amplitude and slope of the initial (1–2 ms) rising phase of the fEPSP were calculated using the acquisition software, and changes in the synaptic response were normalized to the baseline period. De-potentiation was induced by delivery of 1-Hz stimulation for either 5 or 15 min, beginning 5 min following TBS.

Kainic acid administration

In order to investigate how neuronal activity regulates mRNA expression of selected genes we administered Kainic acid (30 mg/kg i.p.) to mice as a single injection to induce profound neuronal activity (seizures). Following injections, the mice were carefully monitored and seizure activity was scored using a standardized seizure scoring scale (Sperk et al., 1985). Only mice that received a grade IV (rearing) or V (falling over) seizure score were included in the subsequent analysis.

Statistics

Data are always presented as the mean value with SEM unless otherwise stated. Comparisons between two groups were performed using unpaired t-tests, if several groups were included a general linear model was used with appropriate levels (for example one-way ANOVA for Kainic acid experiments). For behavioral experiments a generalized estimating equation procedure with an autoregressive covariance matrix or a mixed linear model was used. To further analyze the data, appropriate post hoc tests (with Bonferroni corrections) were used and specific tests were chosen both based on the contrast of interest and the variance structure.

RESULTS AND DISCUSSION

The focus of this thesis has been to study the role of the Nogo-system with a strong focus on NgR1 and its roles in the intact nervous system. We have also analyzed the regulation and expression of the two homologous Nogo-receptors, NgR2 and NgR3 as well as LOTUS, an NgR1 inhibitor. To investigate if NgR1 is involved in memory formation we created a mouse model that overexpresses NgR1 in forebrain neurons (using the CamKII promoter to drive expression), the MemoFlex mouse. Using this mouse model we have investigated how NgR1 overexpression in forebrain neurons (and thereby inability to down-regulate NgR1 when needed) affects the formation and stability of spatial memory and fear conditioning memory. We have also investigated if MemoFlex mice have a different response to drugs of abuse by exposing the mice to a sensitization paradigm. Finally, we have investigated how NgR1 overexpression affects the formation of amyloid plaques by crossing them with a mouse model for Alzheimer's disease.

ACTIVITY-INDUCED REGULATION OF NOGO-RECEPTORS AND LOTUS

It has been known for almost a decade that NgR1 expression can be downregulated by activity and it has also been shown that NgR1 expression is regulated in a variety of different settings (Endo et al., 2007; Josephson et al., 2003; Karlen et al., 2009; Wills et al., 2012). While most evidence suggests that NgR1 is downregulated following situations associated with increased neural activity and plasticity, it was recently shown that NgR1 mRNA and protein was upregulated in a rat stroke model, increasing the complexity of NgR1 expression regulation (Cheatwood et al., 2008). In this study, we subjected mice to Kainic acid and analyzed NgR1 expression and compared results to the earlier publication from our lab (Josephson et al., 2003). We included more time points as well as an analysis of regional levels of mRNA encoding of NgR2, NgR3 and the endogenous NgR1 antagonist LOTUS. We investigated four regions of interest that are all implicated in memory formation and associated with high synaptic plasticity CA1, CA3, the dentate gyrus and the retrosplenial cortex. Hippocampus has long been known to be important for memory and the retrosplenial cortex is also highly associated with both memory and cognition (Vann et al., 2009).

In CA1 of hippocampus the levels of both NgR2 and LOTUS mRNA were highly stable and showed minimal modulation after Kainic acid administration (NgR2 was significantly but minimally downregulated after 12 h). In contrast, NgR1 mRNA was significantly downregulated after 4 h while the expression of NgR3 mRNA was strongly upregulated at 4 h and maintained until 12 h. The expression of LOTUS mRNA was stable in the CA3 region of hippocampus, suggesting that LOTUS levels in hippocampus proper are not significantly regulated by Kainic acid injection. NgR2 and NgR3 mRNA levels were both increased in CA3 at 4-12 h. This upregulation was significant for NgR3 but not for NgR2 (presumably due to high inter-individual variability). The expression pattern of NgR1 in CA3 was very similar to that seen in CA1 and NgR1 mRNA was significantly downregulated at both 2 and 4 h, by 12 h expression had returned to control levels. Interestingly, LOTUS, that did not show any regulation by Kainic acid in CA1 or CA3, was strongly upregulated in the dentate gyrus with a peak in expression seen at 12 h. In addition, both NgR2 and NgR3 mRNA levels

were upregulated in the dentate gyrus, peaking at the 4h mark for NgR3 and at 12 h for NgR2. Compared to NgR2, NgR3 and LOTUS, the levels of NgR1 mRNA expression showed a very different regulation after Kainic acid in the dentate gyrus with a significant decrease peaking already at 2 h. The expression patterns in the retrosplenial cortex were much more stable than those seen in hippocampus and the dentate gyrus. None of the tested genes showed any significant regulation in this area, instead expression patterns were remarkably stable during the 72 h period examined (with the exception of NgR3).

The fact that the regulation of the different Nogo-receptors show temporal and spatial differences is highly interesting, especially as they differ markedly in ligand affinity. NgR2 can bind MAG but not Nogo or OMGP. NgR3, on the other hand, completely lacks affinity to all of the known myelin inhibitors (MAG/OMgp/Nogo) but was recently shown to have affinity for chondroitin sulfate proteoglycans (CSPGs) (Dickendesher et al., 2012). Thus the current knowledge suggest that while both NgR2 and NgR3 only bind to a specific subset of ligands, NgR1 can bind to all of the known myelin inhibitors (MAG/OMgp/Nogo) as well as to CSPGs (Dickendesher et al., 2012). Hence it is possible that downregulation of NgR1 and increased expression of NgR2 and NgR3 will result in changes in how a neurite relates to its local environment through Nogo, MAG, OMgp and CSPGs. Enhanced NgR2 and NgR3 activity could serve to maintain or enhance sensitivity to MAG and CSPGs, while the downregulation of NgR1 could result in a decrease in the sensitivity to Nogo-A (and OMgp); as Nogo-A is also expressed in neurons this could result in a decrease of neuron-neuron inhibition, while myelin-neuron inhibition would remain high, with the result being that local gray matter sprouting could increase without affecting growth of non-terminal axons. The marked and dentate-specific increase of LOTUS mRNA may serve to further block Nogo sensitivity of activated neurites in this region, thus permitting new memories to leave a trace in the hippocampal formation.

In a recent publication it was shown that NgR2 and NgR3 could be downregulated following activation in dissociated hippocampal cell cultures using either (NMDA or KCl) (Wills et al., 2012). The reason for this difference in regulation is not presently known, but could be dependent on either the induction method (NMDA/KCl vs. Kainic acid) or be a due to a difference in response between neurons in cell culture and neurons *in vivo.*

CREATION OF NGR1 OVEREXPRESSING MICE

To investigate if downregulation of NgR1 is of importance in memory formation, we created a mouse with inducible overexpression of NgR1. In the MemoFlex mouse the expression of NgR1 is controlled by the CamKII promoter and utilizes the Tet-off system, hence NgR1 is overexpressed in forebrain neurons and can be turned off by adding doxycycline to the drinking water. Two different lines of MemoFlex mice, that both showed similar but somewhat different overexpression patterns (varying mainly in the intensity of overexpression), were used in subsequent studies. For L1 there was a substantial overexpression of NgR1 seen throughout the cerebral cortex and striatum, including nucleus accumbens. There was also strong transgene expression in hippocampus proper and the neighboring dentate gyrus, as well as in amygdala. Mice from L2 showed similar expression patterns to L1 mice with very strong

overexpression in striatum and the cerebral cortex. The transgene expression in the hippocampus, and dentate gyrus was however a lower than that seen in L1 mice, while amygdala levels were rather high (Fig 1).

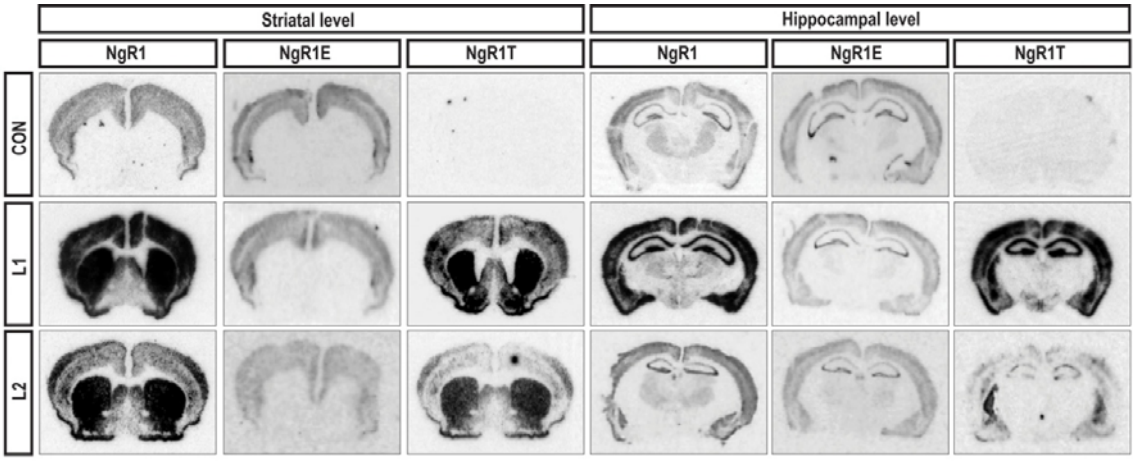
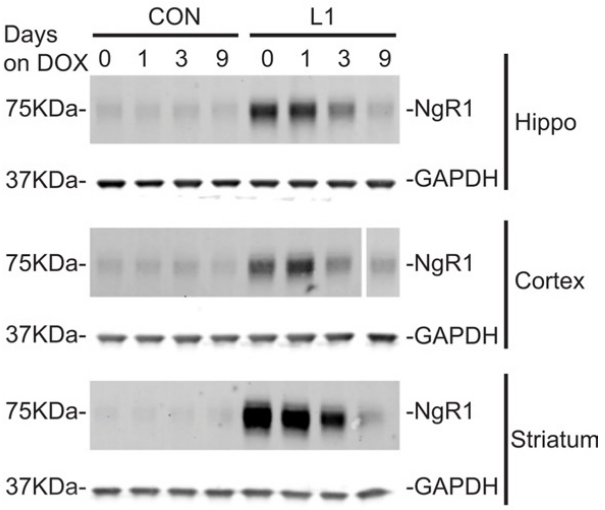


Figure 1. Expression of NgR1 transcripts in MemoFlex mice of Line 1 and 2
Expression is shown at two different levels (striatum and hippocampal). The left column at each level shows total NgR1 levels, the middle column shows endogenous and the right column shows transgene-specific expression.

Figure 2. Protein expression of NgR1 in MemoFlex and control mice
MemoFlex mice of Line 1 have a high over-expression of NgR1 protein. This over-expression can be returned to basal levels after doxycycline treatment for 9 days.



To verify that the increased mRNA expression also correlated with an increase in protein levels we used western blots and could see a strong overexpression of NgR1 protein in the same areas (Fig 2) as seen in the in situ analysis (hence levels of protein and mRNA were correlated). We also wanted to confirm that addition of doxycycline could turn off overexpression and thereby reduce NgR1 levels. As can be seen in Fig 2, already after 3 days of doxycycline treatment the levels of NgR1 had dropped significantly and after 9 days of doxycycline expression, NgR1 expression was similar to that seen in control animals.

MEMOFLEX MICE HAVE NORMAL LOCOMOTOR BEHAVIOR

We next examined the locomotor behavior of MemoFlex mice using two different methods. We first analyzed the coordination and basal locomotor abilities of our mice using the accelerating Rotarod. The time that the mice managed to stay on the Rotarod was recorded and in this test, MemoFlex mice did not show a significant difference in time compared to control mice, suggesting that they do not have impairments in locomotion or in coordination (Fig 3 A). To further analyze their behavior we analyzed basal locomotor activity of MemoFlex and control mice using the AccuScan apparatus. MemoFlex mice performed similarly to control mice (Fig 3 B, C) in this test as well. Hence, overexpression of NgR1 does not appear to affect locomotor activity, coordination or explorative behavior. The fact that basal locomotor function is intact in MemoFlex mice is of profound importance as most memory tests performed on mice requires both locomotion and normal exploratory behavior.

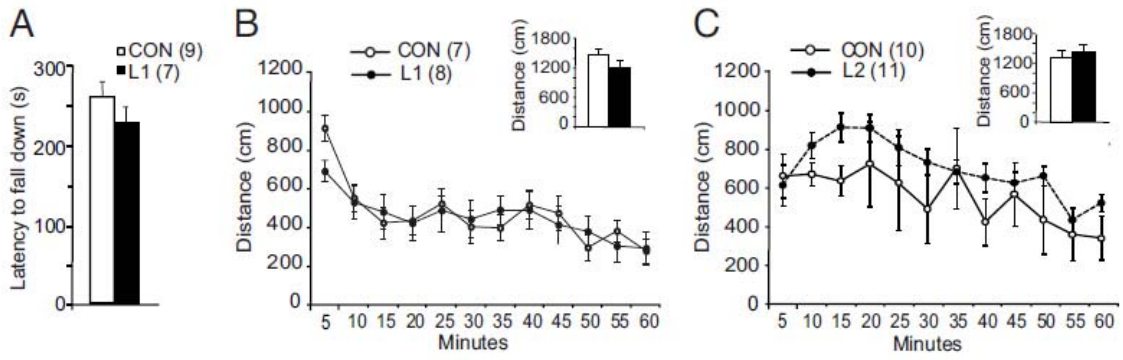


Figure 3. MemoFlex mice show normal locomotor behavior and co-ordination
A, MemoFlex mice were tested in the Rotarod test and showed very similar behavior compared to controls.
B and C, MemoFlex mice had very similar locomotor behavior compared to controls as recorded in activity boxes, suggesting that basic locomotor function and explorative behavior is normal in MemoFlex mice.

DAY TO DAY LEARNING IS INTACT IN MEMOFLEX MICE

To test if NgR1 overexpression would affect memory formation we subjected MemoFlex mice to two different memory tests, the Morris water maze and passive avoidance. The Morris water maze consists of a large circular tank, filled with water, with a submerged hidden platform in one of the four quadrants. Mice will learn to find the platform to escape from the water and the speed with which they find the platform can be used as a measure of their spatial memory skills.

MemoFlex mice from both L1 and L2 performed very similar to controls (Fig 4) and improved their performance in the task in a similar manner as controls. As swim speed can have a profound effect on the performance of mice in the Morris water maze we

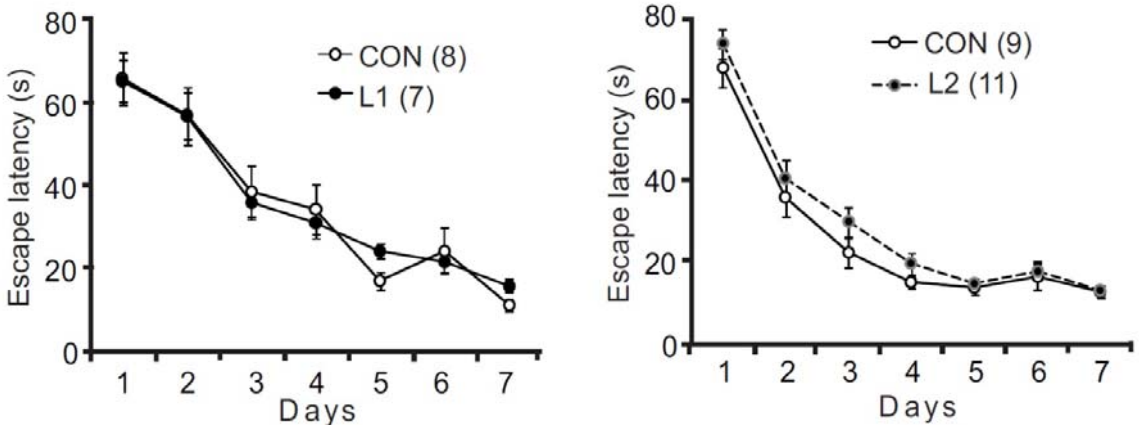


Figure 4 MemoFlex exhibit normal learning in the Morris Water maze
MemoFlex mice could learn the Morris water maze equally well as control mice and showed similar day to day gains.

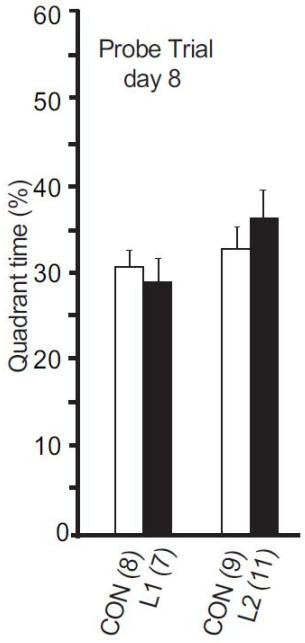


Figure 5. Normal probe trial performances by MemoFlex mice
MemoFlex mice showed similar preference for the platform containing quadrant during the probe trial (platform removed).

compared the swim speed for the two groups and did not find any significant difference between controls and MemoFlex mice. The Morris was intended as a test of hippocampal function but mice can solve the maze using a variety of different strategies. Instead of using a hippocampal based strategy (using the external cues) mice can use a strategy based on procedural memory (swimming at a certain distance from the wall). To control for this, we performed a probe trial at day 8 (platform removed) and analyzed how much time MemoFlex mice spent in the quadrant that used to contain the platform compared to controls (Fig 5). There was no significant difference in the performance between MemoFlex mice and controls, strongly suggesting that MemoFlex mice can learn the Morris water maze equally well as controls and that they learn it using a hippocampus dependent strategy.

We also tested the mice using the passive avoidance test. In this test mice are put into the light compartment of a two compartment box (one light side, one dark side). During the first day the door between the two compartments was closed when the mouse was put into the box and during the first minute the mouse was allowed to explore the light compartment. After 1 minute the door opened and allowed access to the dark side, and when the mouse crossed over (to the dark side) the door closed and a light foot shock was administrated. The mouse was then reintroduced to the chamber at various later time-points and their latency to enter the dark compartment was measured as a score of the strength of the fear memory. When MemoFlex mice and controls were retested again 24h after the training session there was no difference in the performance between control and MemoFlex mice (Fig 6). Supporting the notion that overexpression of NgR1 does not impair the formation of day to day memory. Hence we could show, using two different memory paradigms, that MemoFlex mice are not only able to learn the memory tasks but that they can do so equally well as controls. In fact in almost all tests the performance of MemoFlex mice was remarkably similar to that of controls. We therefore conclude that overexpression of NgR1 in forebrain neurons does not impair day to day memory.

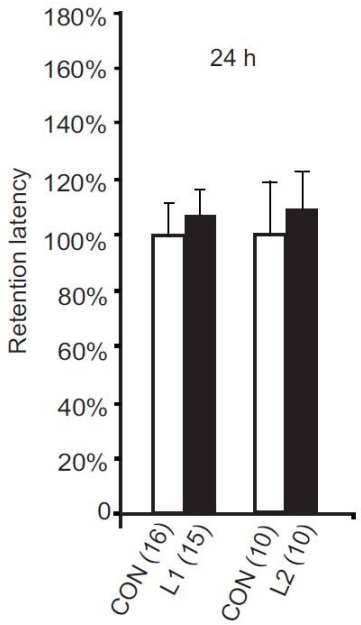


Figure 6. Normal 24 h passive avoidance memory in MemoFlex mice When MemoFlex mice of L1 and L2 were tested in the Passive avoidance test 24 h after training their performance was not significantly different from controls.

MEMOFLEX MICE HAVE NORMAL LTP INDUCTION

We hypothesize that NgR1 should be involved in the synaptic plasticity that underlie long-term memory storage but that this would be on a longer time scale than those events that initiate and maintain LTP. To measure LTP, we recorded extracellular field EPSPs from CA1 in hippocampal slices from control as well as from L1 and L2 MemoFlex mice following 0.033-Hz electrical stimulation. The experiment started with 10 min of stable baseline recordings after which LTP was induced using either high frequency or theta-burst stimulation (Fig 7 A and B) that lasted at least 70 min. No significant difference was seen between MemoFlex and control mice suggesting that NgR1 down-regulation is not required for LTP induction.

To verify that baseline synaptic processes were not affected, fEPSP from control and MemoFlex mice were analyzed and found to have similar time courses and shapes (Fig. 7 A and B), and similar responses across a range of stimulus intensities (Fig. 7 F). Hence it appears that baseline synaptic properties were not altered by NgR1 overexpression.

It is possible that while MemoFlex mice could induce LTP it would not be stable for prolonged time periods. We therefore tested this by following slices for 2h and found stable LTP maintained during this entire period using a single theta burst stimulation, and there was no significant difference between control and L2 mice (Fig. 7 C) ($P = 0.54$, two-way RM-ANOVA, genotype*time).

Loss of NgR1 has been shown to prevent LTD induction in the hippocampus (Lee et al., 2008). As LTD is normally only observed in slices obtained from juvenile animals (Wagner and Alger, 1996) it is unlikely that it would underlie any long-term behavioral changes observed in our adult mice. However, reversal of LTP (de-potentiation) by low frequency stimulation (LFS) is well known to occur in adult animals (Staubli and Scafidi, 1999), and has also been shown to reverse changes in spine morphology induced by LTP (Yang et al., 2008). Therefore we investigated if there was a difference in de-potentiation between MemoFlex and control animals. Two different paradigms (300 or 900 pulses) of LFS (1 Hz) were administrated 5 min after theta burst stimulation and both resulted in de-potentiation of the induced LTP. De-potentiation was dependent on the number of stimuli, 300 (Fig 7 D) vs. 900 pulses (Fig 7 E) but MemoFlex mice did not differ compared to control mice in any of the protocols (300 pulse, $P=0.55$; 900 pulse, $P=0.82$; two-wayRM ANOVA, genotype*time).

These data strongly suggest that MemoFlex mice do not have any significant intrinsic deficits in electrophysiological hippocampal plasticity.

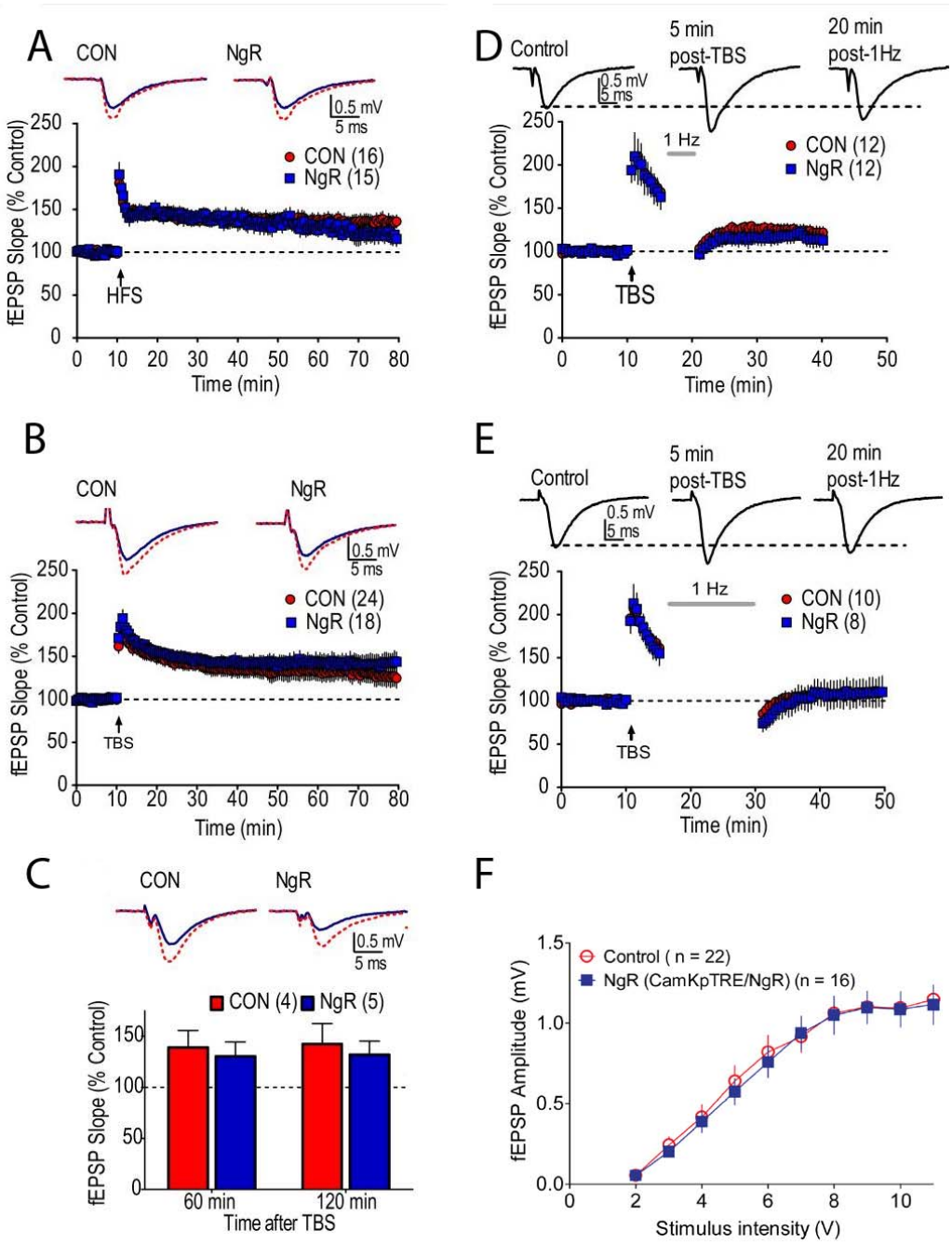


Figure 7. Normal LTP in MemoFlex mice

A and B, LTP induction using HFS or theta-burst stimulation was not changed in MemoFlex mice.

C, LTP was comparable between MemoFlex and controls even at longer time-points

D and E, De-potentiation using 300 or 900 pulses respectively did not reveal a difference between MemoFlex and control mice.

F, Synaptic properties are not changed in MemoFlex mice as tested during a variety of stimulus intensities.

NGR1 OVEREXPRESSION IMPAIRS LASTING MEMORIES

While day to day memory was intact in MemoFlex mice, we hypothesized that lasting memory would be affected by overexpression. We therefore tested the mice that had performed the Morris Water maze in Fig (4) nearly two months after their last training day. In this experiment the platform was at the same position as during the learning phase, hence good performance in this maze would require the mice to remember the position of the platform. Strikingly, MemoFlex mice were severely impaired in this test compared to the control mice and needed significantly longer time to find the platform (Fig 8 B). MemoFlex mice also spent significantly less time in the quadrant containing the platform, again indicating impaired memory of platform location (Fig 8 A).

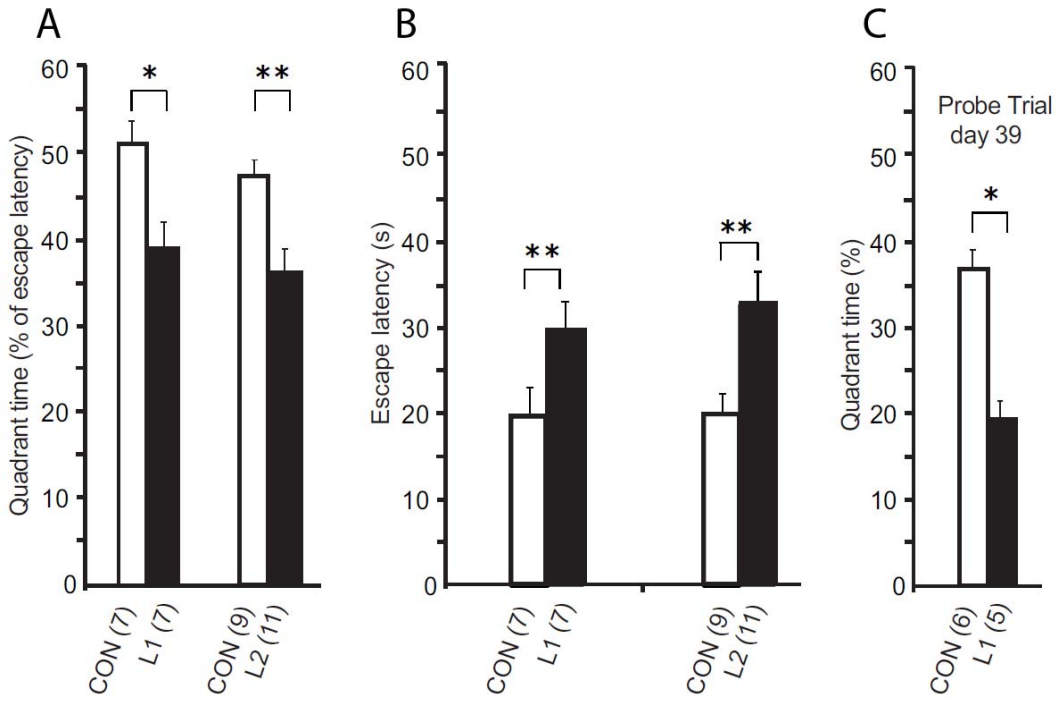


Figure 8. Impaired lasting memories in MemoFlex mice in the Morris water maze
A, percent time spent in platform-containing quadrant 60 days after learning was significantly impaired in MemoFlex mice.
B, the escape latency was increased in MemoFlex mice, at day 60.
C, in a second group of mice a probe trial was performed at day 39 and also in this test MemoFlex mice were significant impaired.

A second group of mice were tested in the Morris water maze with a probe trial at 39 days and performed similarly to the first group of MemoFlex mice, significantly worse than controls (Fig 8 C). Hence while MemoFlex mice are not impaired in their

performance during the learning phase of the Morris water maze their performance is severely impaired after a prolonged waiting period between learning and retest.

We also tested lasting memories in MemoFlex mice that had been trained in the passive avoidance test. As described previously, MemoFlex mice showed similar aversion to the dark compartment as controls, hence there contextual fear condition learning appeared to be normal. When the same animals were exposed to the passive avoidance apparatus after one month the control mice exhibited significantly stronger aversion to the dark compartment than MemoFlex mice (Fig 9 A). To verify that the difference between the groups were specific for NgR1 overexpression and not due to NgR1 induced developmental disturbances we gave a group of mice doxycycline before (3 weeks) and during the experiment, to remove NgR1 overexpression. Both L1 and L2 MemoFlex mice were used in this experiment and both groups now performed similar to control mice indicating that NgR1 overexpression from birth does not result in any major learning disabilities as performance is normalized when overexpression is turned off in adulthood (Fig 9 B). In a second experiment we wanted to further analyze the time window during which NgR1 overexpression impairs formation of lasting memories. To test this, two groups of mice were given doxycycline starting either directly or 7 days after the training day. The passive avoidance boxes used in this experiment were slightly different than those used in the first experiment; instead of

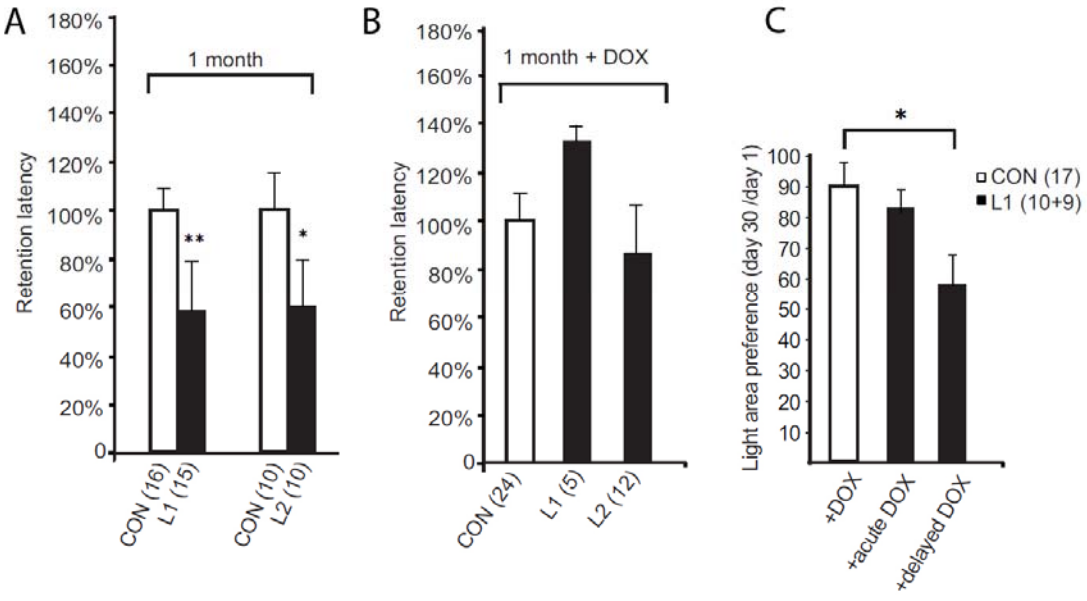


Figure 9. Impaired lasting memories in the Passive avoidance test can be rescued with doxycycline

A, MemoFlex mice were significantly impaired when tested 1 month after the training session.

B, this impairment could be rescued by adding doxycycline to the drinking water 3 weeks before the start of the experiment.

C, the effect could also be reversed by adding doxycycline immediately after the training session but not when it was added after 7 days.

measuring the latency to enter the dark compartment the time spent in the dark compartment was measured. Interestingly, while the group that received doxycycline directly after the training normalized their performance and were not significantly different from controls when tested 1 month after training, MemoFlex mice that received doxycycline starting 7 days after the training phase were still significantly impaired in this test (Fig 9 C).

While NgR1 overexpression does not impair memory formation during day to day learning, the effects on lasting memory are significant and results in a severe impairment. Furthermore, this impairment in memory function can be reversed if doxycycline is given before or at the start of the experiment, indicating that NgR1 overexpression does not result in any major developmental disturbances. However, if doxycycline is given 7 days after the learning event, memory impairments will still be evident suggesting that high NgR1 levels during the first few days following the training paradigm can impair memory formation and that this cannot be rescued by reducing protein levels later on. As discussed earlier, if doxycycline is administrated in the drinking water the overexpression of NgR1 is reduced after 3-9 days and therefore suggesting that NgR1 downregulation is most important rather early after memory induction. Retrograd amnesia after concussion typically spans the last week before the concussion, roughly compatible with the time during which NgR1 down-regulation is needed for lasting memories to become consolidated.

NGR1 AND PLAQUE DEVELOPMENT

It has been shown that NgR1 (Park et al., 2006a; Park et al., 2006b) as well as NgR2 and NgR3 (Zhou et al., 2011) can bind to amyloid precursor protein (APP). However, there are conflicting reports concerning the exact effect of Nogo-receptor interaction with APP. According to the two studies by Park et al (Park et al., 2006a; Park et al., 2006b) NgR1 knock-out mice show an increase in plaque load when crossed with an animal model of Alzheimer's disease (APPswe/PSEN1). Furthermore, when APPswe/PSEN1 mice received soluble NgR1 subcutaneously the plaque load decreased and the impaired performance of APPswe/PSEN1 mice in a radial arm water maze was also attenuated, in fact after the mice started to receive soluble NgR1 their performance improved and moved towards that seen in control mice. A recent study has also shown that NgR1 can bind to APP, but it does so with less affinity than NgR2 and NgR3 and contrary to the studies by Park and colleagues., Zhou and colleagues (Zhou et al., 2011) found that overexpression of Nogo receptors resulted in an increase in Aβ-production not an decrease. Hence while it appears clear that the Nogo-receptors have affinity for APP, their exact function remains somewhat elusive. To investigate this further we crossed our MemoFlex mice (L1) with the same Alzheimer's mouse model (APPswe/PSEN1) as used in the previous studies.

MEMOFLEX/APPSWE/PSEN1 MICE SHOW OVEREXPRESSION OF NGR1

We first wanted to confirm that crossing these two mouse lines would in fact lead to increased expression of NgR1 and that the expression would be strong in the regions that are associated with plaque formation. As expected, crossing MemoFlex mice with APPswe/PSEN1 mice resulted in a strong overexpression of NgR1 in the same regions

as seen in the MemoFlex mouse and these regions are also highly affected by plaque deposition both in humans and in APPswe/PSEN1 mice (Borchelt et al., 1997; Hardy and Selkoe, 2002; Selkoe, 2005). The MemoFlex/APPswe/PSEN1 mouse showed much stronger expression of NgR1 in cortex (Fig 10 A) compared to that seen in the APPswe/PSEN1 mouse (Fig 10 B). The expression was strong and uniform throughout cortex and could be seen in all different cortical layers. NgR1 was also strongly overexpressed in hippocampus and the dentate gyrus (Fig 10 C) and even tough APPswe/PSEN also show robust labeling in hippocampus and the dentate gyrus (Fig 10 D), it was clearly lower than that seen in MemoFlex/APPswe/PSEN1 mice.

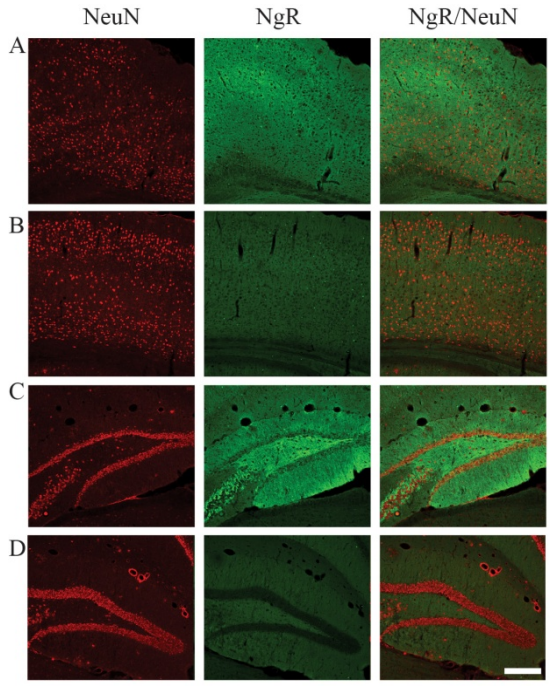


Figure 10. Strong overexpression of NgR1 in MemoFlex/APPswe/PSEN1 mice
A, expression of NeuN and NgR1 in the cortex of MemoFlex/APPswe/PSEN1 mice.
B, the expression of NeuN was similar in APPswe/PSEN1 mice while NgR1 levels are substantially lower.
C, expression of NeuN and NgR1 in the dentate gyrus of MemoFlex/APPswe/PSEN1 mice.
D, again the expression of NeuN was very similar in APPswe/PSEN1 mice but the levels of NgR1 were much lower.

NGR1 OVEREXPRESSION DOES NOT DECREASE PLAQUE

To analyze if NgR1 overexpression influenced the plaque load we stained tissue for Aβ and analyzed the area covered by plaques as well as the number of individual plaques. This was done in two different areas, the cerebral cortex and hippocampus, both regions that show high levels of Aβ in human Alzheimer patients (Hardy and Selkoe, 2002; Selkoe, 2005). We analyzed mice at an average age of 72 weeks and could not find any significant difference in plaque load (Fig 11 A) or in the number of plaques in the cerebral cortex (Fig 1 B). The same analysis was performed in the hippocampus and similarly to the cerebral cortex we could not find any significant difference in plaque load (Fig 11 C) or the number of plaques (Fig 11 D). We also analyzed if the rate of plaque development was different between the two different groups; to do this we added animals with a wider spread in age to the group analyzed previously and plotted plaque load compared to age for MemoFlex/APPswe/PSEN1 and APPswe/PSEN1 mice for the cerebral cortex (Fig 11 E) and hippocampus (Fig 11 F). Both groups were

still very similar and hence we can conclude that neuronal overexpression of NgR1 does not decrease the formation or deposition of plaques in the APPswe/PSEN1 mice.

The fact that very little difference was seen in any measure of the plaques supports the conclusion from Zhou and colleagues that NgR1 does not appear to be a strong effector of Aβ processing. This however does not rule out the possibility that soluble NgR1 could work as a peripheral sink when administrated I.P.

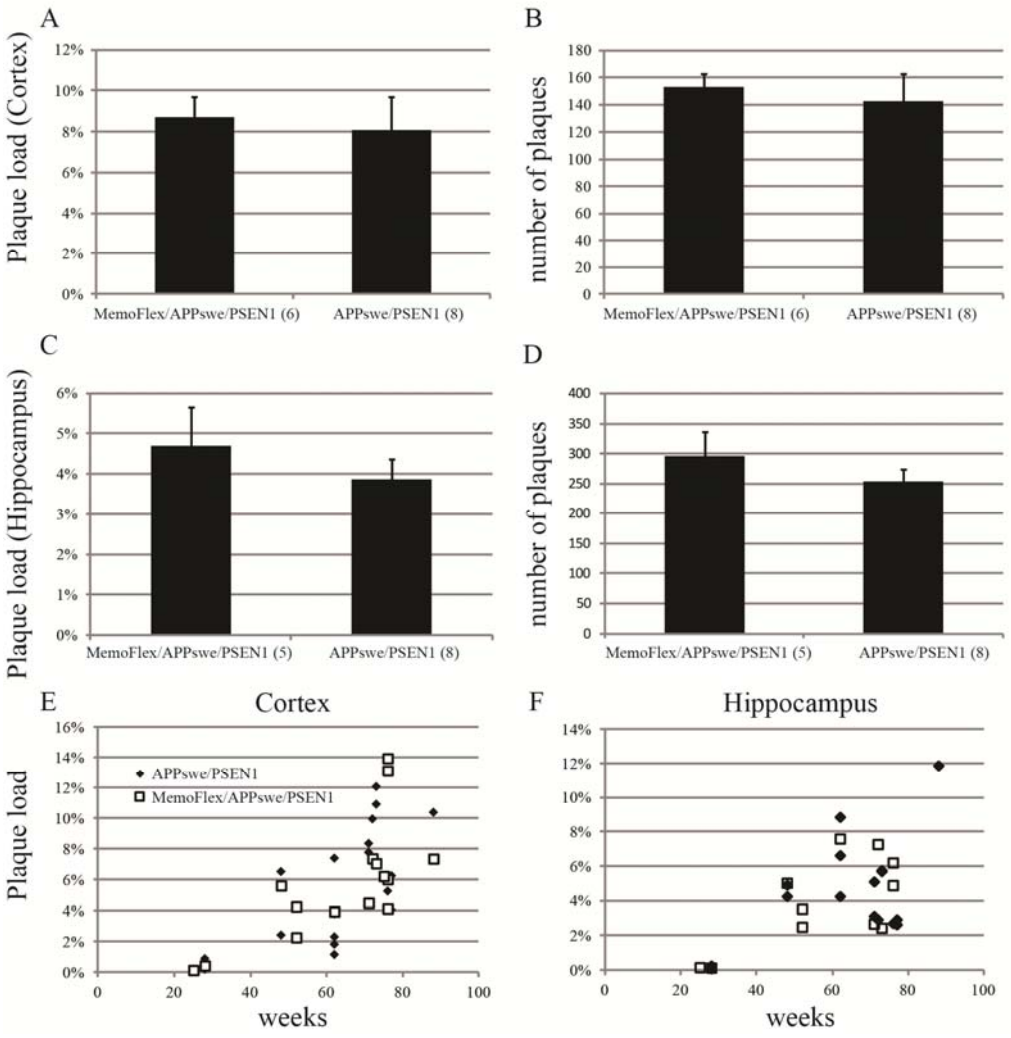


Figure 11. No change in plaque levels in MemoFlex/APPswe/PSEN1 mice
A and B, the area covered by plaques and the numbers of plaques were not significantly different in MemoFlex/APPswe/PSEN1 mice
C and D, Similarly to the situation in cerebral cortex, no significant difference could be seen in the hippocampus
E and F, time-course of plaque development is very similar in MemoFlex/APPswe/PSEN1 and APPswe/PSEN1 mice.

MEMOFLEX/APPSWE/PSEN1 MICE ARE IMPAREID IN MORRIS WATER MAZE

We next investigated if NgR1 overexpression would influence the performance of mice in the Morris water maze. While NgR1 overexpression does not appear to influence plaque load in APPswe/PSEN1, 29 week old MemoFlex/APPswe/PSEN1 mice were significantly impaired (Fig 12 A, Genotype $p = 0.004$, Day $p < 0.001$, and Genotype *Day = 0.252) compared to APPswe/PSEN1 mice in the learning phase of the Morris water maze (Fig 12 A). After the 7 day learning paradigm we also performed a probe trial at day 8 (Fig 12 B) and even though there were no significant difference between the groups, the performance of the MemoFlex/APPswe/PSEN1 mice tended to be worse ($p = 0.096$) suggesting that their knowledge of the platform location could be impaired.

We also retested these mice at an age of 50 weeks to see if the difference would increase with time. As MemoFlex mice have impaired long lasting memory we moved the platform to a new location to minimize any beneficial effects of remembering the old platform location. At an age of 50 week we could see the same trend as previously, MemoFlex/APPswe/PSEN1 had impaired performance in the maze compared to controls (Fig 12 C, Genotype $p = 0.004$, Day $p = 0.001$, Genotype*Day = 0.116). At the probe trial performed on day 8 there was no significant difference between the two groups (Fig 12 D, $p=0.17$)

We next wanted to analyze if the mice were affected differently during the 20 week that separated the 29 and 50 week test. We therefore combined the data from week 29 and week 50 in a single analysis. There was a significant effect of week ($p=0.019$) showing that the animals performed better at week 50 compared to week 29, possibly due to the fact that they were already familiar with the maze. There was also a strong effect of genotype ($p = 0.001$) but there was no significant interaction with genotype (genotype*week $p=0.265$), hence both groups changed in a similar manner with time. We performed the same analysis with regard to the probe trials and in the combined analysis we could see a significant effect of genotype ($p=0.02$) but there was no effect of week ($p=0.865$) and hence performance was very similar on week 29 as it was on week 50 and similarly to what was seen for the learning curve there was no interaction between genotype and week ($p=0.867$).

To confirm that the worse performance of MemoFlex/APPswe/PSEN1 mice was due to cognitive deficits and not due to motor problems, we also analyzed swim speed and thigmotaxic behavior. There was no significant difference in the swim speed (Fig 12 E) between MemoFlex/APPswe/PSEN1 and APPswe/PSEN1 mice at week 29 ($p=0.22$) or at week 50 ($p=0.46$). Both MemoFlex/APPswe/PSEN1 and APPswe/PSEN1 mice showed very similar thigmotaxic behavior (Fig 12 F) at week 29 ($p=0.81$) and at week 50 ($p=0.35$). Therefore it is unlikely that a difference in search strategy would explain the difference that we found between the two groups. Instead it appears that overexpression of NgR1 in APPswe/PSEN1 impairs the spatial cognitive abilities without affecting plaque load or numbers. One possible reason could be that higher than normal expression of NgR1 could result in impairments in compensatory synaptic plasticity (Hashimoto and Masliah, 2003) that is suggested to compensate for the damaged caused by the disease. It has in fact been shown that knocking out Nogo-A can improve performance of APPswe/PSEN1 mice without affecting plaque load presumably trough increasing the ability for compensatory plastic changes (Masliah et al., 2010). Also, while MemoFlex/APPswe/PSEN1 mice did not have significantly higher plaque load in any of the tests, their levels were always higher than that of

controls in all analysis and it is cannot be ruled out that the difference, while not significant, still could have an impact on behavior.

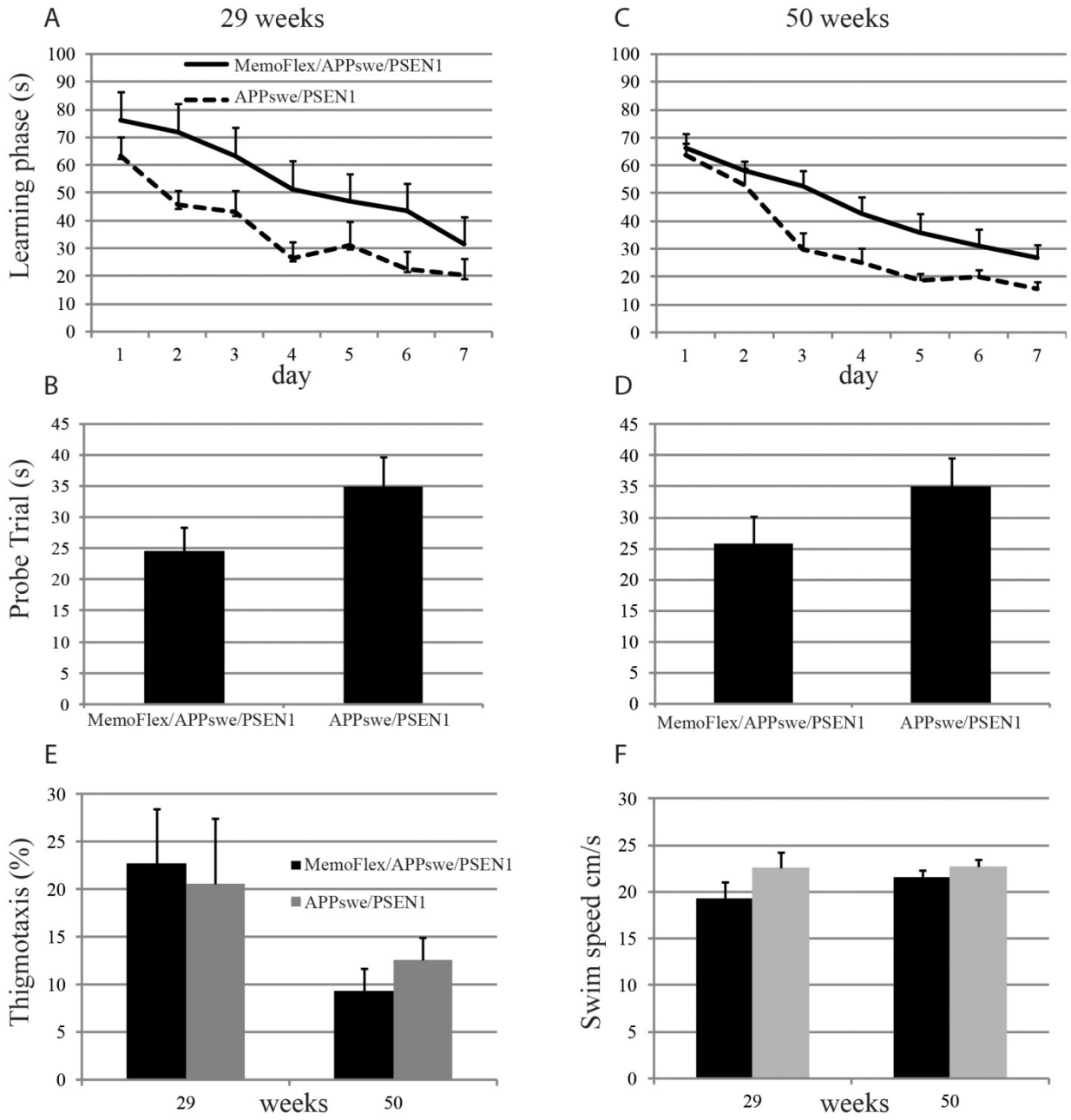


Figure 12. Impaired performance in the Morris water maze for MemoFlex/APPswe/PSEN1 mice

A, MemoFlex/APPswe/PSEN1 mice showed significantly worse learning than controls in the Morris water maze at 29 weeks of age.

B, they also had a tendency of impaired performance in the probe trial.

C, the performance was also impaired at 50 weeks of age.

D, they also showed worse performance in the probe trial but it did not reach significance.

E and F, thigmotaxis and swim speed was not impaired due to overexpression.

NORMAL RADIAL ARM WATER MAZE IN MEMOFLEX/APPSWE/PSEN1 MICE

A second group of animals performed the radial arm water maze (RAWM) instead of the Morris water maze at three different time-points (16, 24 and 28 weeks of age). The RAWM differ from Morris water maze in that instead of a big open platform the RAWM consists of a small open center area with 6 arms protruding from it. In one of the 6 arms there will be a hidden platform and the other 5 arms will serve as starting arms. There was no significant difference between the two groups of animals in any of the 3 different time points tested (Fig 13 A-C), 16 weeks (Fig 13 A, genotype $p = 0.102$, day $p < 0.001$ genotype*day $p = 0.977$), 24 weeks (Fig 13 B, genotype $p = 0.963$ day $p < 0.001$, genotype*day $p = 0.307$) and 28 weeks (Fig 13 C, genotype $p = 0.248$, day $p < 0.001$ and genotype*day $p = 0.307$).

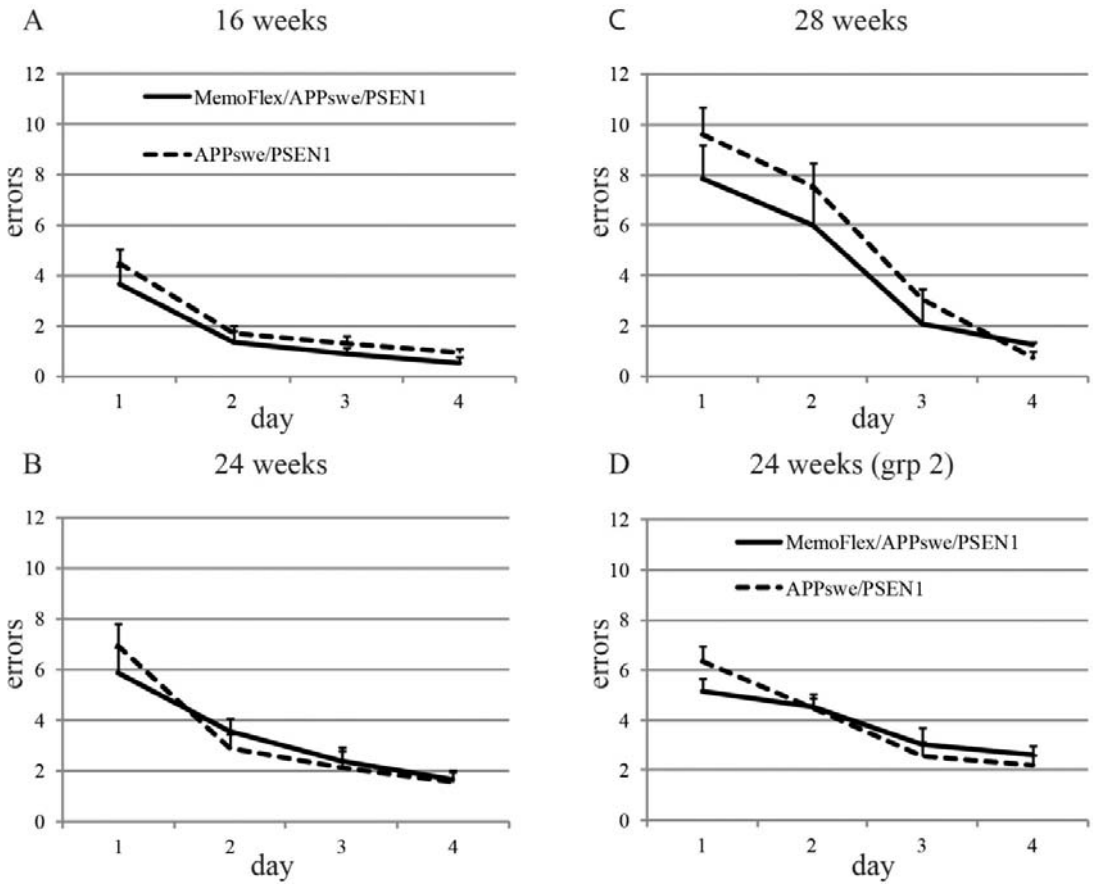


Figure 13. Normal performance in the Radial arm water maze for MemoFlex/APPswe/PSEN1 mice

A, B, C. The performance for MemoFlex/APPswe/PSEN1 mice was very similar to that of APPswe/PSEN1 mice during all three different time points. Even though both groups performed worse with time, they did so at a similar rate.

D, a second group of mice were also tested in the radial arm water maze was also in this test the performance of MemoFlex/APPswe/PSEN1 mice was indistinguishable from that of APPswe/PSEN1 mice.

Even though there was no significant difference between the two groups of mice their performance significantly worsened with time (week $p < 0.001$ and week*day $p < 0.001$) when all of the time-points were analyzed together. However, both groups of animals had similar change with time and there was no genotype*week effect ($p = 0.674$)

We also tested a second group of mice (those that later performed the Morris water maze) and similarly to the first group there was no significant difference in their performance (Fig 13 D) compared to controls (genotype $p = 0.875$, day $p < 0.001$, genotype*day $p = 0.214$). The reason for the significant difference seen in the Morris water maze could either be that it poses somewhat different demands on the mice than the RAWM and/or that the mice that performed the Morris water maze where older and it is possible that behavioral differences would occur with time also in the RAWM.

MEMOFLEX MICE SHOW INCREASED SENSITIZATION

As we have previously shown that NgR1 overexpression impairs the formation of lasting memories of both spatial and fear based types, we wanted to investigate if the role of NgR1 could be even more general. A second type of long term adaptation that can occur in the brain is that caused by drugs of abuse. Once a person becomes addicted to a drug of abuse, the addiction will generally be lifelong. Hence, whatever changes that occur must be very stably stored in the brain and also be rather substantial. One of the most well studied paradigms of the effects of drugs is a process called sensitization. When animals are repeatedly injected with psycostimulants such as amphetamine, their response will increase with time (Segal and Mandell, 1974), even when the dose is held constant. Thus their response becomes sensitized and these effects can be very stabile, lasting over a year in rodents and for several years in monkeys (Castner and Williams, 2007). We therefore wanted to investigate if overexpression of NgR1 would affect the long lasting stability of sensitization.

We began by subjecting MemoFlex mice and controls to a 9 day sensitization paradigm. In our setup mice first received saline injections for 2 days to establish a baseline and after the initial two days they received amphetamine injections for 7 consecutive days Fig 14 (A). The performance of MemoFlex (L1) and control animals were similarly during the first 2 days of saline injections. However, during the 7 days of amphetamine administration a significant difference in the development of the sensitization response could be seen. MemoFlex mice became significantly more sensitized compared to controls (Genotype $p= 0.5$, Day < 0.001 and Genotype*Day=0.017). While control mice level off after 3-4 days of sensitization, MemoFlex mice continue to increase their locomotor activity throughout the experiment. To further analyze the difference in sensitization behavior we also analyzed the amount of time the mice spent moving (Fig 14 D) and this correlated very well with the distance moved. Hence the difference was due to an increase in moving time and not a difference in movement speed. One factor that can greatly influence the amount of locomotion animals exhibit is amount of stereotypies (repetitive motions). This was analyzed and both groups exhibited similar levels of stereotypies and it is therefore unlikely that the level of stereotypy would significantly affect the difference that was seen between the groups in the development of sensitization.

We next wanted to see if the difference in sensitization could be rescued by adding doxycycline to the drinking water (starting three weeks before the start of the experiment). When doxycycline was added and hence NgR1 levels normalized, then so did the sensitization behavior (Fig 14 B) and the behavior of MemoFlex mice was not significantly different from that of controls. As seen in the previous groups, locomotor behavior correlated very well with movement time (Fig 14 E), suggesting that movement speed was very similar between the different groups. There was no significant difference in the amount of stereotypies either (Fig 14 H).

As an increase in the amount of NgR1 expression resulted in an increase in sensitization, we wanted to see if lower levels of NgR1 would result in a decrease in sensitization. We therefore subjected a group of NgR1 knockout mice (NgR1-/-) to the same sensitization paradigm with heterozygote NgR1 mice (NgR1+/-) as controls. There was no significant difference between NgR1-/- and NgR+/- mice during the sensitization paradigm. Instead, they performed very similar with regard to both distance (Fig 14 C) and movement time (Fig 14 F). There was no significant difference in the amount of stereotypy between the groups and even if the levels were somewhat higher in the NgR1-/- group, it is unlikely that this would mask a significant difference in locomotor behavior. It is thus reasonable to assume that loss of NgR1 have small if any effects on the development of a sensitization behavior.

It thus appears that overexpression of NgR1 results in a large increase in sensitization because MemoFlex mice are unable to counteract sensitization and such that the amphetamine responses level off after 3-4 days as is normally the case. This difference is due to the expression of NgR1 protein during the sensitization paradigm and not due to developmental disturbances as doxycycline treatment during the sensitization paradigm normalizes the behavior of MemoFlex mice. Finally, the fact that complete loss of NgR1 does not affect the development of a sensitization, suggests that activation induced by amphetamine is strong enough to cause a maximal plastic response even in the presence of normal NgR genes, and that thus loss of NgR1 does not give any additional plastic benefits in this case.

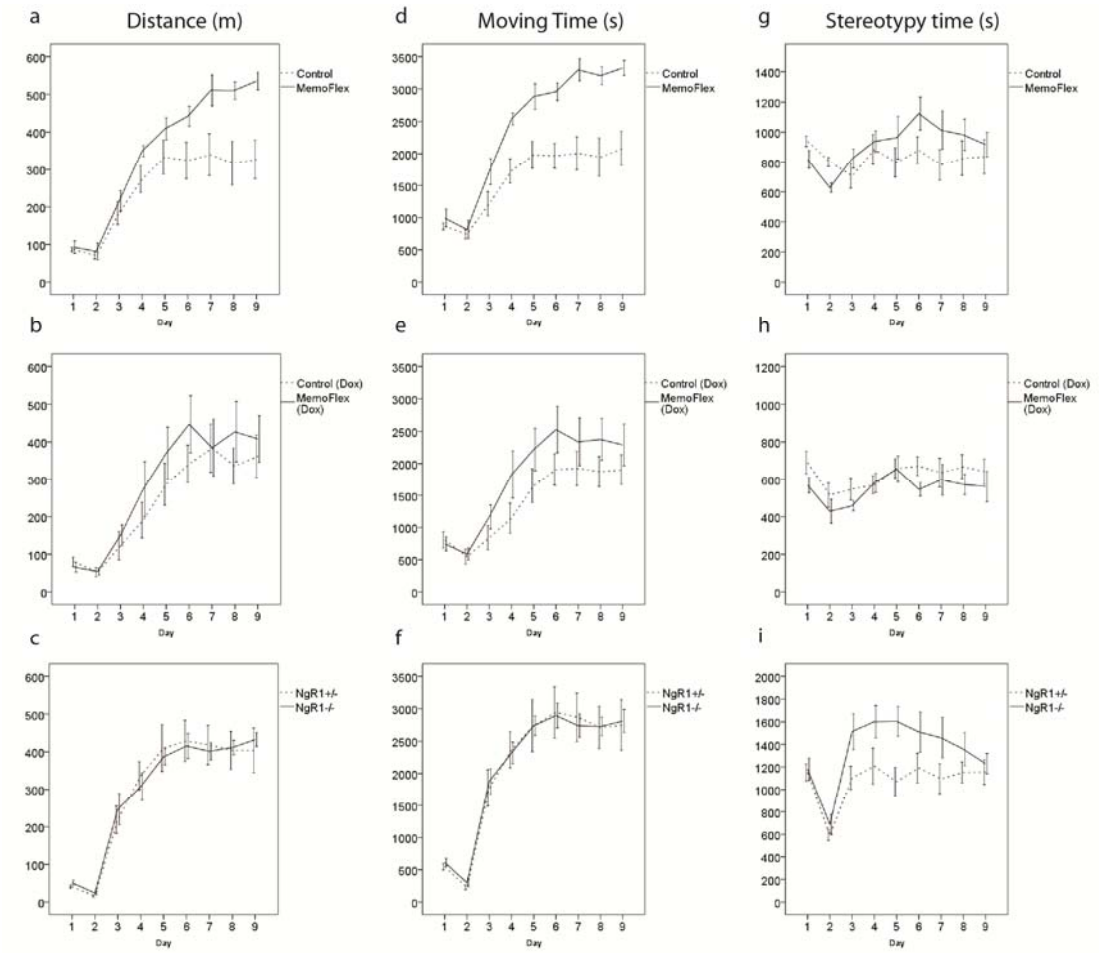


Figure 14. MemoFlex mice have increased sensitization compared to controls that can be normalized with doxycycline

A, D, G MemoFlex and control mice received saline for the first 2 days followed by amphetamine (2mg/kg) for 7 days and locomotion (A), moving time (B) and stereotypy times (C) were measured. MemoFlex mice showed increased sensitization but comparable stereotypy.

B, E, H when doxycycline was added to the drinking water the performance of MemoFlex mice normalized

C, F, I the sensitization of NgR1-/- mice was not altered compared to NgR1+/- mice.

LONG TERM SENSITIZATION IS REDUCED IN MEMOFLEX MICE

As the important effects of NgR1 overexpression has been seen on lasting memory, we wanted to see if the effect of the locomotor sensitization would decrease with time in MemoFlex mice while remaining stable in control mice. We therefore re-exposed the mice to amphetamine 1 and 3 months after the last day of sensitization. MemoFlex mice showed a significant decrease ($p=0.041$) in their locomotor behavior with time (Fig 15 A) while control mice had a tendency increased locomotor response with time ($p=0.52$ last day to 3 months later).

The mice that performed the sensitization experiment on doxycycline were kept on doxycycline and re-tested 1 and 3 months after the end of the sensitization paradigm. For both groups there was no significant effect of time. After one month the performance was very similar for both groups and after 3 months a small but non-significant drop was found in the MemoFlex mice and the tendency for an increase that was seen in the controls without doxycycline (Fig 15 B) could not be seen. It therefore appears that giving mice doxycycline largely stabilizes the long term sensitization but that some residual impairment might still be found. It is also possible that the long treatment time with doxycycline (around 4 months in total) could have an effect in itself.

NgR1+/- and NgR1-/- mice showed very similar long term response to amphetamine exposure but the changes seen were larger in NgR1-/- compared to NgR1+/- mice (Fig 15 C). NgR1-/- mice exhibited a significant increase in their sensitization response when re-exposed to amphetamine 1 month after their last injection, but when retested 3 months after the learning phase their sensitization level was back to that seen immediately after the end of the sensitization paradigm. NgR1+/- mice had a stable response to the sensitization paradigm and did not show any significant change with time.

We conclude that NgR1 overexpression impairs lasting memory function in a wide array of different settings and that removing NgR1 overexpression in adulthood largely normalizes the mice with respect to memory function. In contrast, while NgR1-/- mice have been shown have increased plasticity, for instance a monocular deprivation task (McGee et al., 2005) it is possible that NgR1 levels are normally held and regulated in a way that does not require additional downregulation to function satisfactorily. It is however interesting that NgR1-/- mice showed an increase in sensitization after 1 month that later reverted at 3 months. It is possible that NgR1-/- mice indeed are more plastic in this setting and that the high plasticity could also enhance extinction or relearning.

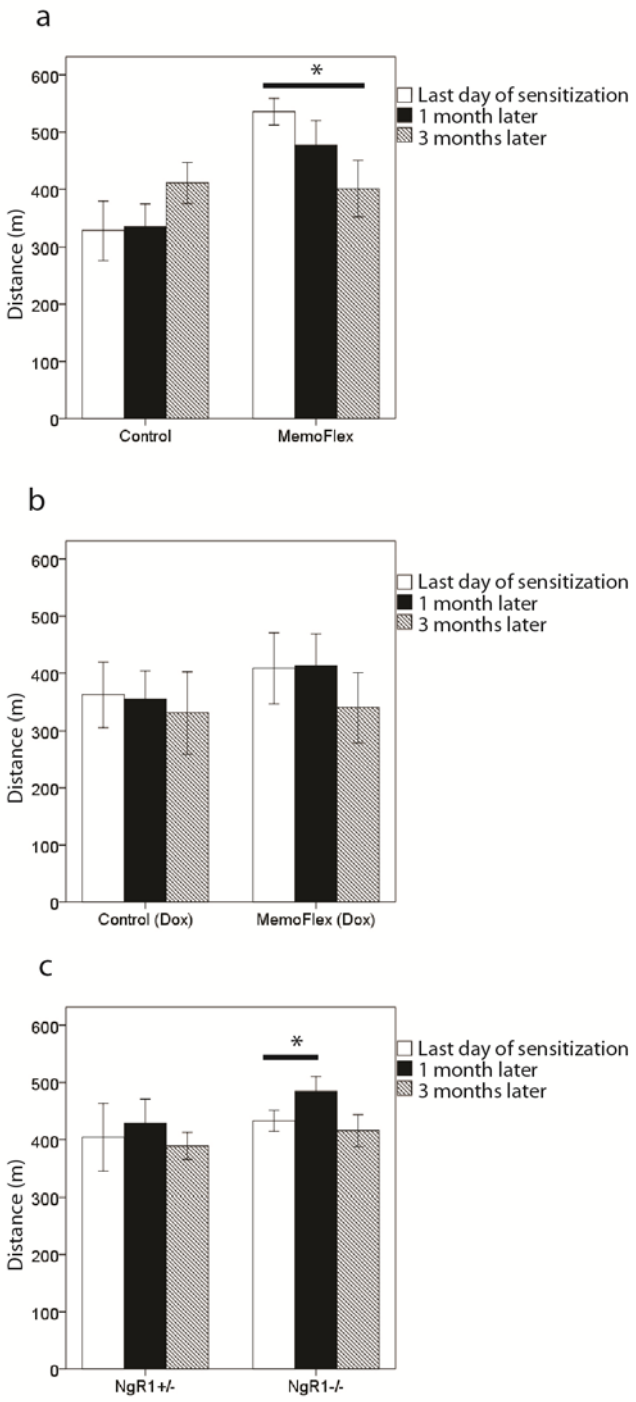


Figure 15. Impaired long term sensitization in MemoFlex mice
A, MemoFlex mice showed a decrease in their sensitization from the last day of sensitization until they were retested 3 months later.
B, MemoFlex mice with doxycycline did not show any significant change in sensitization over time but the inter-animal variance was also high.
C, NgR1-/- mice had a significant increase in sensitization 1 month after the sensitization paradigm but this disappeared before the 3 month time point.

NGR1 OVEREXPRESSION AFFECTS SPINE DYNAMICS

The development of a sensitization behavior is closely linked to changes in spine structure and density (Luscher and Malenka, 2011; Robinson and Kolb, 2004; Russo et al., 2010). We therefore wanted to evaluate if NgR1 overexpression would affect spine density following amphetamine treatment.

To investigate how NgR1 overxpression would affect spine density following amphetamine exposure, we subjected mice to 7 daily injections of amphetamine (same amount of amphetamine as during the sensitization experiment). We first analyzed the spine density at the distal part of the apical dendrite of pyramidal neurons in the cingulated gyrus. Control mice showed a tendency of an increase in spine density of

around 10% (p=0.079) while MemoFlex mice instead showed a significant decrease in spine density (p=0.003) following amphetamine treatment (Fig 16 A). To further analyze how NgR1 affected spine morphology, we also analyzed the number of thin (Fig 16 B) and mushroom spines (Fig 16 C). Amphetamine resulted in a significant decrease in thin spines for MemoFlex mice (p=0.006) on amphetamine compared to saline treated MemoFlex mice. For mushroom spines there was no significant effect of either treatment or genotype (Fig 16 C).

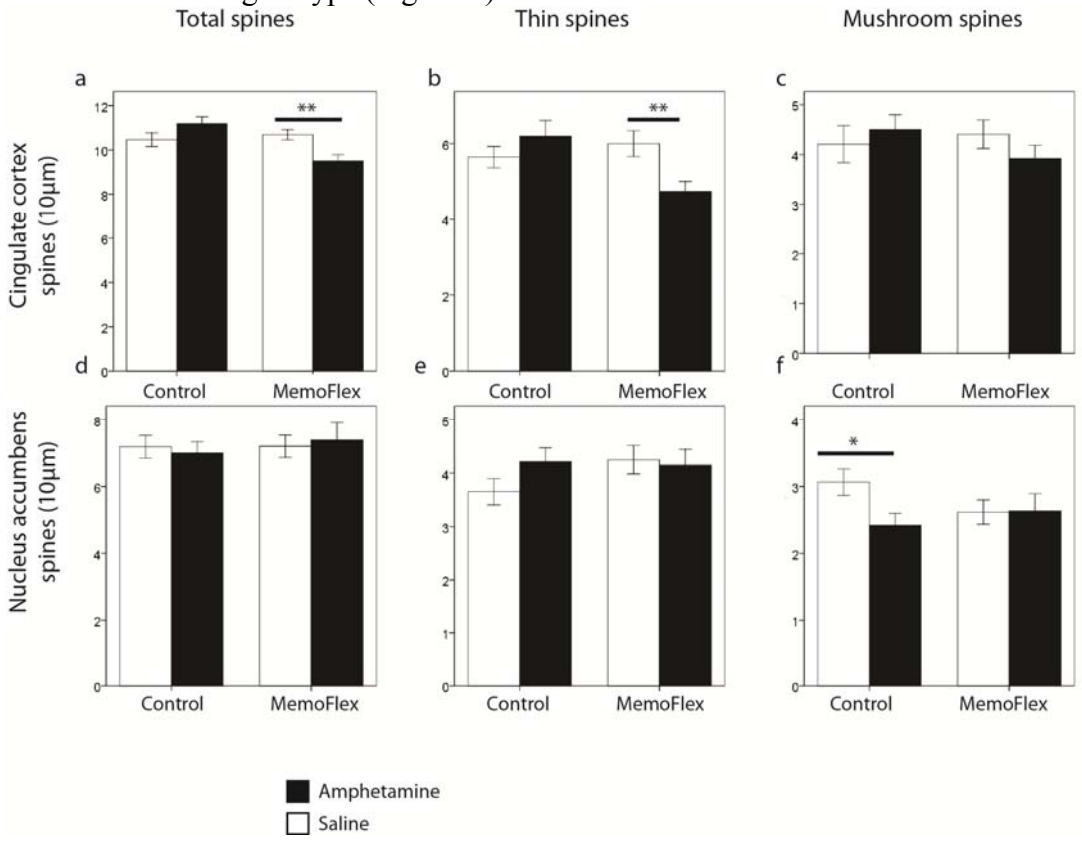


Figure 16. Overexpression of NgR1 affects spine dynamics following amphetamine sensitization

A, B, C control mice showed a tendency to an increase in spine density in the cingulated gyrus while MemoFlex mice instead had significantly decreased spine density (A). When analyzed per subtype it was shown that this was mostly due to a decrease in thin spines (B) while mushroom spines were more stable.

D, E, F in nucleus accumbens the spine densities were very stable after amphetamine injections (D). Looking at spine subtypes we could see a significant decrease in densities of Mushroom spines in control mice while MemoFlex mice had very stable spine densities.

We also analyzed the spine density on medium spiny neurons in the shell of nucleus accumbens and could not see any effect on spine density after amphetamine in control or MemoFlex mice (Fig 16 D). However, when we analyzed thin (Fig 16 E) and mushrooms spines (Fig 16 F) individually, we could see that control mice had a decreased number of mushroom shaped spines following amphetamine treatment (p=0.04). The mean amount of thin spines did increase following amphetamine treatment of control mice, but this was not significant, most likely the reason why there

was no effect on overall spine density. In nucleus accumbens spine density and subtype distribution was minimally affected by amphetamine for MemoFlex mice; possibly due to the fact that NgR1 overexpression locked those synapses in place or that NgR1 function in nucleus accumbens is of less importance. When looking at the synapse distribution after saline injections (that should reflect the normal spine levels in these animals) the cortical patterns were very similar between MemoFlex and controls, while there is a tendency of more thin and less mushroom spines in striatum.

The difference regarding the effects on NgR1 on the spine changes in cerebral cortex are highly interesting. It is well established that exposing animals to repeated injections of amphetamine results in changes in spine morphology and density (Luscher and Malenka, 2011; Robinson and Kolb, 2004; Russo et al., 2010). However, synaptic plasticity does not only involve the formation of new synapses, elimination of synapses is also of profound importance and while increased spine density is often seen in the early phases after an event that enhances synaptic plasticity, spine number can decline back towards baseline levels without the loss of the memory. Hence, it is possible that during a learning event far more new spines will sprout than what is necessary for forming the engram and through a selection process only those that form functional connections will be saved and the others will be removed. Hence, instead of seeing memory encoding as an increase in spine formation, it could be seen as a period of increased turnover of spines. In such a setting, increased levels of NgR1 could decrease the ability to form new spines but keep the elimination side of the equation equal or even increased and thereby resulting in spine loss.

When it comes to the spine changes seen in nucleus accumbens, the effects seen in our control mice might at first appear paradoxical. The establishment of new memories is generally believed to be in part due to the enhancement of connections between neurons and due to strengthening (growing) of spines so that thin spines should be converted to thicker more influential spines. With this in mind, it is surprising that we see a reduction in the density of mushroom spines in control mice. However, development of sensitization is not a one-way street and it is likely that there are changes occurring that both enhance and inhibit the establishment of a sensitization behavior. In fact it has been shown in several studies that sensitization is followed by a period of LTD induction in synapses from the prefrontal cortex in the nucleus accumbens (Brebner et al., 2005; Goto and Grace, 2005; Thomas et al., 2001) and perhaps this could explain why our control mice level off after a few days of sensitization while MemoFlex mice continue to sensitize for the entire sensitization period.

CONCLUDING REMARKS

The aim of this thesis has been to further characterize the potential role of the Nogo-system as a regulator of plasticity with a focus on memory systems. We have shown that all three Nogo-receptors are regulated by activity in areas of importance for memory function (hippocampus). When NgR1 is overexpressed (thereby removing the ability to downregulate NgR1), mice have normal day to day memories in both Morris Water maze and in passive avoidance tests. Strikingly, when overexpressing mice were compared to controls after a long waiting period (> 1 month) their performance was significantly impaired. Thus, while inability to downregulate NgR1 in forebrain neurons does not impair 24 h memory, such memories do not lead to normal lasting memories, suggesting a key role for NgR1 in the consolidation of recently acquired memories. This effect appears to be caused by NgR1 overexpression just around the learning event, as silencing of the transgenic NgR1 overexpression (using doxycycline) just after the learning event could rescue their behavior, while later silencing (after 7 days) did not.

NgR1 mice exhibit an enhanced sensitization response to amphetamine, compared to controls. When overexpression was removed their performance became similar to that seen in controls, indicating that NgR1 overexpression can also influence this response. However, sensitization was not affected in NgR1-/- mice. When sensitization was retested at later intervals NgR1 overexpressing mice showed a reduction of sensitization, supporting the notion that Ngr1 overexpression impairs lasting memory. Overall, NgR1-/- mice were very similar to controls, suggesting that removal of NgR1 has limited effects on sensitization behavior. We also analyzed spine structure on pyramidal neurons in the cingulate gyrus and on medium spiny neurons (MSN) in the shell of nucleus accumbens. In cortex, there was a significant reduction of spine density in MemoFlex mice while control mice exhibited a tendency of increased spine density. A possible explanation for the reduction in spine density could be that NgR1 overexpression increases the likely-hood of spine collapse so that in times of a needed increase of plasticity (such as during the sensitization phase), the ratio of formation and elimination could be altered, leading to a net spine loss. In striatum, spine levels in medium spiny neurons where remarkably similar for NgR1 overexpressing mice, while control mice showed significant alterations. This suggests that NgR1 overexpression reduces synaptic plasticity in medium spiny neurons.

We also investigated if NgR1 overexpression would have an effect on plaque formation and found that even though plaque levels were similar to controls, the overexpression of NgR1 caused these mice to perform worse in the Morris water maze. A possible reason for this deficit could be that NgR1 overexpression reduces the amount of compensatory plasticity that can otherwise occur in response to the increasing plaque load, and thus aggravate symptoms.

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